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PRELIMINARY EXPERIMENTS ON THE RELATION OF GROWTH-PROMOTING SUBSTANCES TO THE REST PERIOD IN FRUIT TREES

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(with one figure)

Introduction

Prolonged rest of leaf and flower buds of fruit trees occurs commonly in mild climates as in the western and southern states. It is especially evident in springs following mild winters, but may be nearly absent in springs following more severe winters.

Similarly it has been found that the rest period can be artificially prolonged by keeping plants under warm conditions in a greenhouse or can be artificially broken by placing the trees in cold storage (**2**). Many other treatments have been used for breaking the rest, but the action of these diverse agents has given no clue to the nature of the physiological factors responsible for the rest condition. Work on inhibition of lateral buds on plants has shown that the growth-promoting hormone, auxin, is a controlling factor. There are several reasons for believing that also in the resumption of growth by buds of trees, growth-promoting substances are involved. In this article results are presented of preliminary experiments undertaken from this viewpoint.

Experimentation

Determination of auxin in lateral buds of pear and cherry

The presence and distribution of auxin in lateral buds of trees has been studied by Zimmermann (**5**). The change in auxin concentration in terminal buds of apple during the growing season has been followed by Avery, Burkholder, and Creighton (**1**). In the present work, resting lateral buds in which no hormone could be obtained by the *standard Avena* technique, were studied by means of the *deseeded Avena* test method (**4**). In November, buds from pear and cherry trees growing in the orchard were tested for auxin. The buds were cut at the base of the scales, placed on wet

filter paper for 10 to 20 minutes and then individually on standard 2 per cent. agar blocks for two hours. The blocks were then tested by the standard and also by the *deseeded Avena* method. No trace of *active auxin* was found. At this time, two-year-old cherry and pear trees were placed in cold storage at a constant temperature of 2° C. and comparable trees were moved into a greenhouse at a minimum temperature of approximately 15° C. At intervals buds from each of a number of previously selected comparable branches on the different trees were tested for the presence of auxin.

The results are summarized in table I. For small concentrations of auxin, curvatures reach a maximum not later than 4 to 5 hours after the blocks are applied. After longer times of application, traces of activity comparable to those produced by substances which can be converted into auxin were obtained. It can be seen that in the first four tests no curvatures characteristic of auxin were obtained. The next three experiments indicate that such a substance accumulates in the buds in cold storage but not in those in the greenhouse. The later experiments show that after an extended exposure to low temperature either in cold storage or in the field the buds are able to cause relatively large characteristic auxin curvatures in deseeded plants and also give significant curvatures in standard tests. From buds in the greenhouse, however, no response could be obtained. Soon after the appearance of auxin an increase in size of the buds was observed. In the tests made March 4 and 5, cherry buds from cold storage had leaves protruding about 1 mm. above the scales. The buds of cherry and pear from the field, tested March 4, were slightly more developed, as were the cherry buds from cold storage in the last test, May 3. It is realized that many metabolic changes occur during breaking of the rest period. The liberation of auxin in the buds may be one result of these changes essential for resumption of growth.

Breaking of the rest period by added growth–promoting substances

The results presented above indicate a possibility of breaking prolonged rest periods of buds by the administration of growth-promoting substances. Resting one-year-old pear, apple, and peach trees grown continuously in a warm greenhouse were used. Tips were removed from selected branches and dilute aqueous solutions of the substances listed in table II were passed into the trees through the cut ends. The effect of the treatments on the development of buds was noticeable two to four weeks after application. The final observations, April 1, are summarized in table II. It can be seen that on trees receiving no treatment a few buds grew on two pear trees, one on two apple trees, and none on peach trees. In trees injected with water, usually one to three buds next to the cut end on the injected branch grew, but not vigorously. In trees injected with DN, tryptamine, or vitamin B_1 the number of developing buds was no greater than in those injected only with water. But the application of vitamin B_1 possibly caused more vigorous development

TABLE I

AMOUNTS OF AUXIN, IN DEGREES, OBTAINED FROM LATERAL BUDS PLACED ON AGAR BLOCKS FOR 2 HOURS, AS DETERMINED BY DESEEDED *Avena* METHOD. EACH FIGURE REPRESENTS THE MEAN OF TEN TO TWELVE BUDS. THE NEGATIVE VALUES REPRESENT AUXIN ACTIVITY

DATE	TIME OF TEST IN HOURS	CHERRY			PEAR			AGAR BLOCKS
		BUDS FROM TREES IN			BUDS FROM TREES IN			
		GREENHOUSE	COLD STORAGE	FIELD	GREENHOUSE	COLD STORAGE	FIELD	
11/23/36	2	+1.2	+ 1.6		−0.3	+ 1.8		
	14	−2.0	− 3.0		−2.2	− 1.2		
11/29/36	S.T.*	+	+	+	+	+	+	
12/1/36	5			+ 1.2			+0.1	
	5			+ 1.1			.....	
	18			− 3.0			−2.2	
	18			− 3.2			.....	
12/9/36	15	−0.2	− 0.4		−0.1	+ 0.6		
12/23/36	18	−1.6	− 6.2		−3.8	− 4.5		+0.1
1/18/37	17	−0.5	− 5.5		−2.3	− 7.1		+1.5
2/12/37	18	.....	− 4.0		.....	− 4.0		+2.0
3/4/37†	5			−17.7(1)‡			−18.8(2)‡	
	5			−23.9(2)			−10.7(3)	
	5			−13.6(3)				
3/5/37	18	−0.2	− 2.6	− 3.0	−2.2	− 3.2	−7.3	
	18	+0.2	− 3.3	− 2.0	−2.5	− 5.4	−7.5	+0.9
	S.T.	+0.2	− 4.5	− 3.2	.....	− 5.6	−6.1	
	S.T.	+1.5	− 3.0	− 4.3	−2.0	− 9.2	.....	
3/11/37	18	+2.8	− 2.9	− 6.3	−2.3	− 4.5	−3.9	
	S.T.	+1.5	− 2.3	− 1.3	+0.8	+ 0.8	+2.5	
5/3/37	3.5	+1.4	−24.2		−0.2	− 4.8		
	24	0.0	.....	.....		−17.3		
	S.T.	+3.5	−12.4	.....		− 9.0		

* In standard test, (S.T.) blocks are applied 110 min.
† Buds had grown to lengths of 0.5 to 0.8 cm.
‡ (1), (2), (3) refer to time in hours buds were left on agar blocks.

TABLE II

EFFECT OF VARIOUS SUBSTANCES ON GROWTH OF DORMANT BUDS

TREES OBSERVED			SUBSTANCE APPLIED	SUBSTANCE AMOUNT	No. BUDS	No. BUDS GROWNG APRIL 1	REMARKS
Pear 12/1	Branch	1	Yeast ext. 1	0.0 cc.	31	2	Only apical buds grew
"	"	2	" "	0.1 "	42	4	
"	"	3	" "	0.5 "	36	21	4 buds near top developed shoots
"	"	4	" "	2.5 "	15	10	
"	"	5	" "	10.0 "			Branch killed but many buds on trunk above and below grew
Pear 2/22	Tree	1	Yeast ext. 2	2 cc.	175	49	Growing buds distributed over whole tree—12 on trunk
"	"	2	" "	2 "	149	37	Growing buds distributed over whole tree—13 on trunk
"	"	3	Vitamin B_1	1 mg.	191	2	Only apical buds on injected branch grew
"	"	4	" "	1 "	137	4	" " " " " " "
"	"	5	*D N	0.5 "	191	6	Growing buds on upper branches, 1 on trunk
"	"	6	Water		182	3	Apical buds on injected branches grew
"	"	7	0		128	1	
"	"	8	0		174	6	3 buds grew on trunk
"	"	9	Heteroauxin	1 "	124	8	Growing buds on injected or adjacent branches
Apple 2/19	Tree	1	Yeast ext. 2	2 cc.	51	30	Best growth on lower part. Upper part of tree injured
"	"	2	" "	2 "	50	6	Only lower buds grew. Upper part of tree killed
"	"	3	Vitamin B_1	1 mg.	50	1	Growing bud near top
"	"	4	" "	1 "	50	3	" buds " "
"	"	5	Water		48	2	" " " "
"	"	6	0		50	0	
"	"	7	0		49	1	Growing bud near middle of trunk

* Dinitro-ortho-cyclo-hexyl-phenol.

TABLE II—(*Continued*)

Trees observed		Substance		No. buds	No. buds growng April 1	Remarks
		Applied	Amount			
Peach						
1/5	Tree 1	Yeast ext. 2	8 cc.	54	34	4 branches injected 2 cc. each; lower buds grew best
"	" 2	" "	10 "	29	19	5 branches injected 2 cc. each; lower buds grew best
"	" 3	" "	20 "	32	14	Injected through top of trunk. Upper branches and trunk killed
"	" 4	" "	8 "	95	40	4 branches injected, 2 cc. each
1/20	" 5	Water	.....	30	2	3 branches injected, about 10 cc. each
"	" 6	Tryptophane	1 mg.	45	8	Growth of buds weak
"	" 7	Tryptamine	1 "	74	0	
2/19	" 8	Vitamin B_1	1 "	28	2	Apical buds grew weakly
"	" 9	" "	1 "	39	2	" " " "
2/22	" 10	Heteroauxin	1 "	54	21	Buds grew from tip to base of injected branch, moderately strong growth
"	" 11	0		420	0	

of the buds that grew. Tryptophane was applied to peach trees only. It caused weak growth of a number of buds along the injected branch. β-indoleacetic acid (heteroauxin) produced moderate growth of buds from tip to base of injected branches and some development near the bases of lower adjacent branches in peach trees, while in pear trees it caused moderate development of a few scattered buds on injected and adjacent branches.

The best development of buds was obtained by the injection of a preparation obtained by a series of extractions of autolyzed brewers' and bakers' yeasts. On treated peach trees the buds grew vigorously along the injected branches and on the lower parts of adjacent branches. In the treated apple trees which were relatively small and had only a few branches, the dosage was too large, so that the upper portions of the stems were damaged. Below the damaged regions, however, buds developed along the entire stems. On pear trees (fig. 1) the development of buds extended from the point of injection throughout the entire tree.

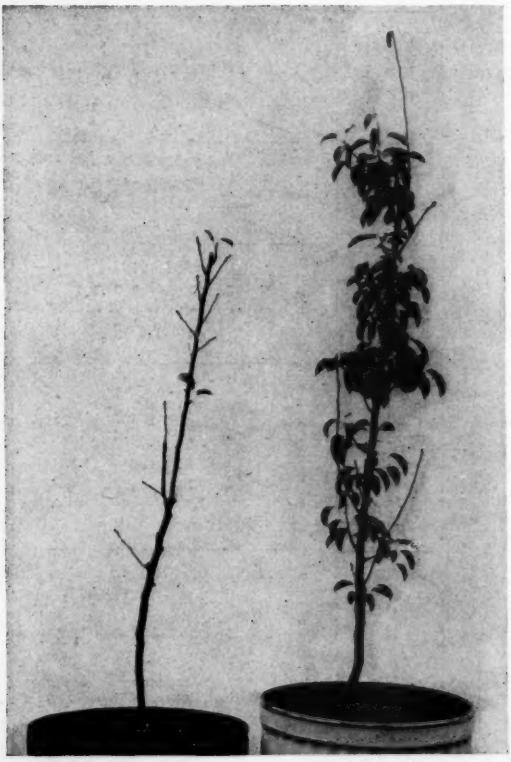

FIG. 1. The effect of injected yeast extract on Bartlett pear trees. Left, untreated; right, treated.

On the peach trees the developing shoots grew to lengths of 2 to 12 cm., on the pear trees, to lengths of 4 to 12 cm. and then ceased growing. On the apple trees, however, elongation continued until the shoots were 25 to 50 cm. long.

Discussion

The results of these experiments demonstrate again that low temperature, either in the field or in cold storage, is essential for normal breaking of the rest period. As a result of exposure to low temperature a precursor of auxin accumulates in the buds, followed by the gradual appearance of auxin. Its appearance in the buds is correlated with ending of the rest. Our results are in striking agreement with the work of MIROV (3) who demonstrated that in resting seeds of sugar pine no active auxin is present, but that upon stratification in cold storage, auxin gradually appears in considerable concentrations, and that parallel with its appearance the seeds are able to germinate. The results presented here also show that rest can be broken by the administration of certain growth-promoting substances. Further experiments on a larger scale than as yet has been possible are necessary to determine the active component from yeast, and to determine the relative effectiveness and relationship of the different active substances. Merely arbitrary dosages were chosen above. The fact that heteroauxin in these experiments was found to stimulate the growth of resting buds is not to be considered contradictory to its known inhibiting action on bud development under different physiological and experimental conditions.

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PLANT DIASTASE IN EVIDENCE AS TO THE FORMATION AND STRUCTURE OF STARCH GRANULES[1]

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(WITH SEVEN FIGURES)

Introduction

In a recent paper (3) evidence was presented as to the existence of two distinctive sugar-forming plant diastases. These diastases occur together in certain cereals and in various other plants; but in many plants only one of them is apparent. No reference was made in that paper to a third type predominately liquefying, which accompanies these two and which is essential to the purpose which they serve. This latter diastase can best be understood by stating the characteristics of the other two.

Investigation

In a study of different parts of the wheat grain, weighed quantities of finely powdered material of each part were digested in water at 20° C. for 1 hour. Starch paste was then added, and the diastase was allowed to act upon it during the following hour. A plain 2 per cent. Lintner starch paste, pH about 6.2, was used in comparison with a similar paste buffered with sodium acetate and acetic acid to a pH of about 4.5. The maltose formed by the diastase in the flour was materially increased in the more acid starch over that in the plain starch. Bran from the same wheat gave opposite results, except at lower temperatures. The results from the bran of this wheat after germination were like those from the bran of ungerminated wheat but were more pronounced.

Further investigation showed that these conditions were caused by two distinct types of diastase. The type predominating in the flour is more active in acid media, so that when the quantity of maltose produced in the more acid starch (pH about 4.5) is divided by that produced in the less acid starch (pH about 6.2) the ratio is greater than one. With the type which predominates in bran, however, the reverse is true.

Studies carried out under various conditions showed that 60° C. is an appropriate temperature to use to determine which of these two types of diastase predominate in any plant substance under investigation. Table I shows an example of the different activities found in different parts of the wheat grain at different temperatures, and with contrasting pH. It is here shown that both flour and bran contain much diastase. The relative amounts present cannot easily be stated because of differences in the conditions under

[1] Presented, in substance, at the meeting of the American Society of Plant Physiologists at Atlantic City, December 31, 1936.

which they give best results. The diastase in the germ is of the type found in the bran but it occurs in distinctly smaller amounts.

TABLE I

DIASTATIC ACTIVITY IN PARTS OF AN UNGERMINATED KANSAS WHEAT*

PARTS OF WHEAT	TEMPERATURE OF ACTIVITY				
	20° C.		60° C.		
	KIND OF STARCH				
	PLAIN	BUFFERED	PLAIN (A)	BUFFERED (B)	100 × RATIO B TO A
	pH OF SUBSTRATE				
	6.2	4.5	6.2	4.5	
	MALTOSE				
	mg.	*mg.*	*mg.*	*mg.*	
Flour	185	280	71	148	208
Bran	106	122	121	68	56
Germ	28	28	83	23	28

* Fifty mg. of each finely ground material digested in 5 cc. water at 20° C. for 1 hour. Added to each portion at the desired temperature, 50 cc. of 2 per cent. Lintner starch paste. Mixture digested for 1 hour. Maltose calculated to mg. in each tube.

Following the methods just mentioned, a survey has been made of the diastase in a very wide range of plant substances. The results are such as to indicate the presence of a uniform system throughout the vegetable kingdom. Determinations have been made on the carefully separated parts of newly formed and immature seeds. Diastase was nearly always found in considerable quantities. In some specimens (*e.g.*, rice) the amount was too small to be determined without the use of an inconveniently large quantity of material. In a large number of varieties of wheat and closely related cereals there was evidence of both types of diastase. In general the distribution was as in the mature and germinated grain. The one type predominated in the starchy endosperm, the other in the bran.[2]

Among germinated grains the two types showed clearly in wheat, barley, and rye. The results with oats, Indian corn, buckwheat, and others indicated the bran type only. Where both types of diastase are present, the sugar formed at any one point as fixed by pH and temperature is a resultant of the combined action of the two. The significance of this in determining the diastatic value of malt is apparent.

[2] Flour and starchy endosperm are here used interchangeably. In these separations, as in wheat milling, the aleurone layer, classed as part of the endosperm, always forms part of the bran.

Soy bean material was examined under many conditions. In all stages the flour type of diastase predominated in the cotyledons. The bran type was present in the skin. This was most readily observed in the immature beans. The bran type was also found in the pods and leaves.

Many roots, tubers, and bulbs show the bran type of diastase. A few showed the flour type to predominate. The edible sweet potato showed the flour type in a marked degree. Young shoots from the germinating sweet potato, and later the fully developed leaves, showed the bran type. The amount of diastase is very considerable in both the sweet potato and its shoots.

Leaves from many species of flowering plants selected to cover the entire range of both monocotyledons and dicotyledons showed the bran type of diastase to predominate in all of them. Taka-diastase from molds, and industrial diastase from bacteria, also showed these properties. This bran type of enzyme appears to predominate wherever active vegetation is taking place, and in most cases to such an extent as to exclude the other type. Because of this fact it has elsewhere been called *vegetative diastase*. At the same time, the type which predominates in flour and in certain other reserve material was called *reserve diastase*.

The comparison of these diastases has been extended much beyond the methods here described. In a recent series of experiments the predominating diastase in the flour of ungerminated wheat and the predominating diastase in the bran of germinated wheat were prepared in a greater degree of purity by fractional precipitation with alcohol from water extracts of the flour and bran. Solutions of these diastases in water were combined with 2 per cent. pastes from raw potato starch. Successive portions of this paste had been brought to different pH by equal quantities of sodium acetate and suitable quantities of acetic acid or decinormal sodium hydroxide.

To contrast the action of these diastases, the amounts of maltose produced at 60° C. in different portions are plotted in figure 1. Previously comparisons had been made with other and like precipitates using Lintner starch paste. In general the results were as here shown, but somewhat modified because of the partial removal of the starch coating by acid used in the preparation of that type of starch.

A study of vegetative diastase has been greatly facilitated by a liberal sample of bacterial diastase[3] sufficient for a long period of use. In this study, results have been obtained showing its activity at different temperatures in starch pastes of different pH and under other conditions. These studies included fractional modification or destruction of parts of the diastase by heat.

Figure 2 shows the relation of this bacterial diastase to the diastase of flour at 60° C. in pastes of varying pH. The graphs are similar to those in

[3] Kindly supplied by Wallerstein Laboratories, New York.

figure 1 with diastase separated from water extracts of flour from ungerminated wheat and from the bran of germinated wheat. Here the bacterial diastase, like that from the bran, gives abundant maltose in starch of the higher pH, and in a restricted amount in starch of the lower pH.

The temperature range of activity of this bacterial diastase is found to extend from about 0° C. to the local boiling point of water (97° C.) with a maximum activity between 50° and 60° C. The pH range in the more favorable temperature is from about 3.8 to 7.8. The optimum pH in the more favorable temperatures is about 5.2 to 6.2 with a tendency toward the latter

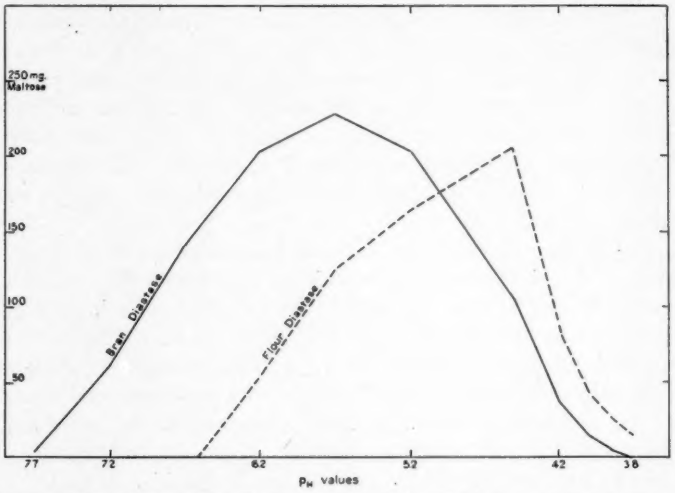

FIG. 1. Maltose formed in 60 min. at 60° C. by diastase precipitated from water extracts of bran of germinated wheat and flour of ungerminated wheat. Substrates were 50-cc. portions of 2 per cent. pastes of raw potato starch, with ten different pH values obtained by buffering with sodium acetate, acetic acid, and decinormal sodium hydroxide. Values given are as found at 25° C. Reduction was determined with potassium ferricyanide, using BLISH and SANDSTADT tables (1).

value. At about 65° C. there is a rapid falling off in the amount of sugar produced and a steady decrease in activity from this temperature upward.

The optimum conditions for the activity of liquefying diastase are so similar to those for this latter sugar-forming diastase that actual separation of the two is difficult. Their properties and differences have been compared by other means. When a suitable amount of liquefying diastase is present there is at suitable temperature and pH a rapid clearing of the opalescence of starch paste. With the bacterial diastase used, even as little as 1 mg. in water added to 50 cc. of paste containing 1 gm. of raw starch will, under

suitable conditions, become clear within one-half minute. The optimum temperature for this clearing is from 70° to 80° C. with a pH of about 6.8. In all of the many tests made under different conditions, this change has been accompanied with the production of a small amount of sugar. The liquefying diastase of germinated wheat and other cereals was found to have properties similar to those of the diastase from bacteria, but specimens from these sources seemed to be less suited to detailed study.

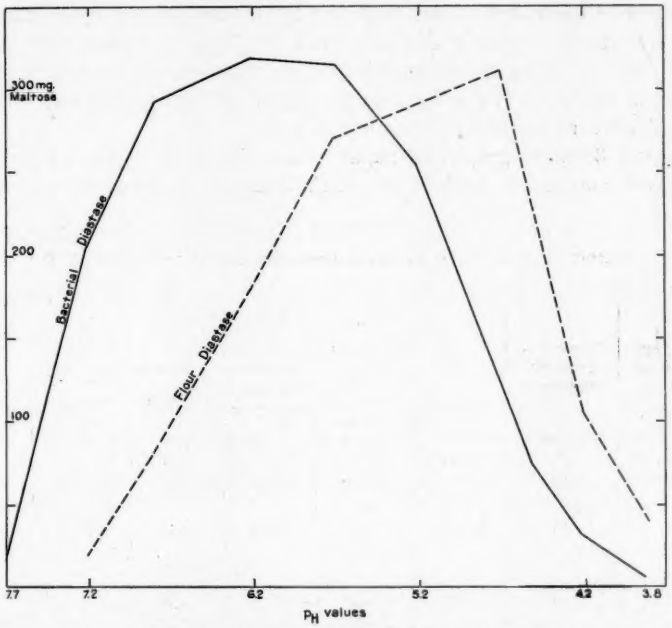

FIG. 2. Maltose formed by 1 mg. bacterial diastase and diastase in water extract from 100 mg. flour of ungerminated wheat. Other conditions as for figure 1. Note similarity of graph for bacterial diastase and for precipitated diastase from wheat bran.

With 5 mg. of bacterial diastase used in a 2 per cent. paste of 1 gm. of raw potato starch (pH 6.8), the liquids cleared in one-half minute. At six temperatures differing by 10 degrees each, the amount of reducing substance produced, calculated as maltose, did not in any case exceed 2 per cent. of the original starch. At 97° C. the clearing was complete in three-quarters of a minute. If longer time is allowed for the clearing, it will take place after the diastase has been in boiling water for several minutes. This emphasizes the resistance of the liquefying diastase to heat. When heated in water solution at 90° C. for 10 minutes, then added to 2 per cent. paste of raw potato

starch at 40° and 50° C. and the liquid held at these temperatures, the formation of sugar proceeded slowly for many days. The sugar first formed gave phenyl-hydrazine reaction for maltose. In later stages, reaction for dextrose was obtained. The clear blue test with iodine obtained in the early stages of this reaction gradually gives way to violet, red, and yellow.

Bacterial diastase was heated in water for 10 minutes at 85° and 90° C. Equivalents of 7½ mg. each were then added to several 50-cc. portions of 2 per cent. unbuffered paste from raw potato starch, pH about 6.8, and kept at 50° C. In three hours, 95 per cent. and 90 per cent. of the opalescence had disappeared with reduction equivalent to 7 and 5 mg. of maltose respectively. At 20 hours, solutions were completely clear, with reduction equal to 55 and 30 mg. of maltose. Reduction steadily increased, and at 312 hours showed an equivalent of 415 and 385 mg. of maltose.

Figure 3 shows graphs of sugar production with 1 mg. of unheated diastase in comparison with 50 mg. of the diastase heated at 80° and 85° C.

TABLE II

ACTIVITIES OF BACTERIAL DIASTASE AFTER HEATING IN WATER AT 95° C.*

DIASTASE MINUTES HEATED	TEMPERATURE OF DIGESTION	HOURS DIGESTED					
		40 HOURS			64 HOURS		
		MALTOSE	CONDITION OF LIQUID	IODINE REACTION	MALTOSE	CONDITION OF LIQUID	IODINE REACTION
min.	°C.	*mg.*			*mg.*		
5	40	40	Clear	Blue	58	Clear	Blue
8	40	31	Faint Cloud	Blue	44	Clear	Blue
11	40	22	Faint Cloud	Blue	30	Clear	Blue
5	50	60	Clear	Blue	87	Clear	Violet
8	50	51	Clear	Blue	96	Clear	Violet Blue
11	50	23	Faint Cloud	Blue	33	Clear	Blue

* Three lots of 250 mg. bacterial diastase heated in 100 cc. water for 5, 8, and 11 minutes, cooled and 3-cc. portions added to 50 cc. of 2 per cent. paste from untreated potato starch. This is equivalent to 7½ mg. of diastase to 1 gm. of starch. One series was digested at 40°C. the other at 50° C. Solutions were examined at intervals. Examples given show the amounts of maltose formed in 40 and 64 hours, with other observational data.

for 10 minutes. The pH value of the substrate was about 5.2 at 25° C. but it changes consistently with the temperature. The change in relative positions of the graph lines with these changes is significant. Near 65° C. they cross each other. The sugar formed by 50 mg. of the liquefying diastase which has survived heat for 10 minutes at 80° C. is now distinctly higher than from the smaller amount of liquefying diastase in the 1 mg. of unheated material.

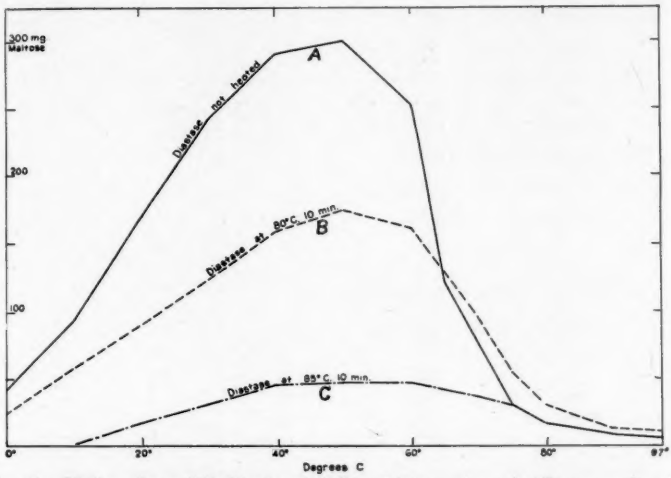

FIG. 3. Maltose formed in 60 min. at different temperatures in 50-cc. samples of 2 per cent. raw potato starch paste, pH 5.2 at 25° C. A = 1 mg. diastase not heated. B = 50 mg. diastase from 1 gm. heated in 100 cc. water at 80° C. for 10 min. C = 50 mg. diastase from 1 gm. heated in 100 cc. water at 85° C. for 10 minutes. Boiling point of water was 97° C.

On the other hand the 1 mg. of unheated diastase produced a very much larger proportion of sugar below the critical temperature of 65° to 70° C. than was produced by 50 mg. of the heated diastase. Similar changes were found in all of a large number of determinations under different conditions. With 50 mg. of diastase heated to 85° C. the sugar formation at the higher temperatures still equals that from the 1 mg. of unheated diastase, but in this case there is much less sugar formation at the lower temperatures than with either of the other portions. These data, in connection with other information, indicate the presence of two types of diastase in this group: a predominantly sugar forming type and a predominantly liquefying type.

The problem of the starch granule, its structure, and its formation is intimately associated with the problem of diastase. A study of the diastases sheds new light on both problems. By use of the diastases the obscurity which surrounds the formation of starch granules has been removed and

something of what lies beneath has been observed. Figure 4 shows a series of stages in the breaking down of natural, uncooked potato starch under the slow action of bacterial diastase; figure 5 shows potato starch in formation; and figure 6 shows some instructive miscellaneous potato starch formations. Numerous photographs were made of selected specimens as they lay scattered among others under the microscope. For repeated examinations, the micro-

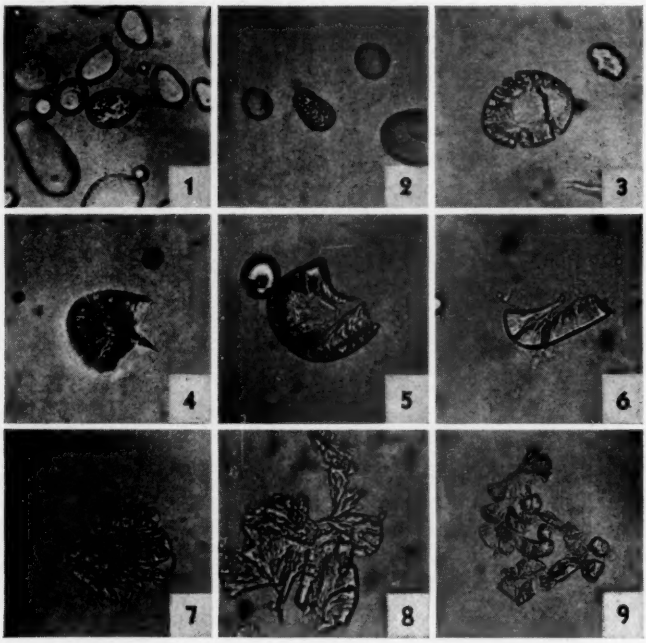

FIG. 4. Potato starch disintegrated by bacterial diastase. 1, coating partly removed. 2, coating completely removed. 3, granule much swollen showing break at right angles to longer axis. 4, group of segments forming long point. 5, segments loosened and spread. 6, smaller group showing long segments. 7, enlarged granule showing loosened segments closely grouped. 8, segments of tree-like formation. 9, smaller segments.

scopic slides with the starch in the solutions of diastase were kept moist in a covered petri dish at a temperature somewhat below the gelatinizing point of the starch. Vegetative diastase, whether obtained from bacteria, from germinating seeds, or from other materials, is similar in its action.

The numbers in parentheses in the following paragraphs correspond to the numbers (1 to 27) in figures 4 to 6. At first the outer coating of the starch grain is eaten away in patches (1). When this is completely removed,

the remainder of the granule presents a surface of jagged points suggesting an Osage orange (2). Gradually the granule becomes enlarged from the absorption of liquid (3). In many cases numerous spaces are seen between the points, leaving a deeply serrated edge. Frequently a crack appears across this enlarged granule and at right angles to the main axis. The parts

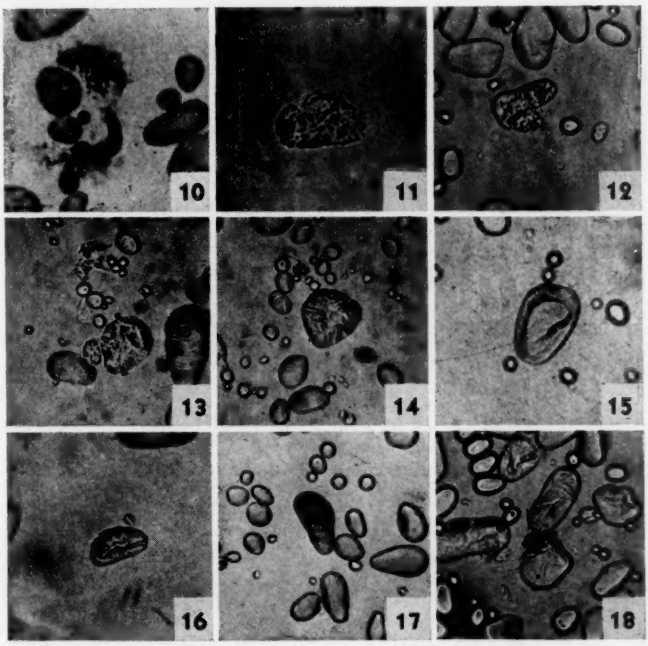

FIG. 5. Potato starch in formation. 10, wisps of loose starch substance lightly stained with iodine. 11, more concentrated aggregate of starch substance. 12, another aggregate with slight coating at bend. 13, circular granule with light coating. 14, group of aggregates with light coating. 15, well-shaped granule with coat forming from border toward center. 16, coating extending from border toward center. 17, coating more nearly complete; granule more coated and blued with iodine. 18, group of completed and nearly completed granules, one at center with new starch aggregate near end.

separate. One shows a long point (4, 23, 24), the other a funnel-shaped opening from which the point was withdrawn (24).

As these parts disintegrate they are seen to have been made of series of small segments. Some are long, straight, and narrow (6). Others are bent, with the broader end abutting the surface at nearly right angles (5). The narrow end of these is directed toward the so-called point of growth and the tip of the long projection first mentioned (4) appears to stop at that point.

Those segments from the end nearest the point of growth are short, showing triangular or wedgeshaped outlines (9). Sometimes the segments show a treelike formation (8). These may separate and become much enlarged as by the absorption or imbibition of water. Many segments are seen with channels through them, and appear to be tubular in form. This is sometimes due to spaces between two or more of them, but even very minute single seg-

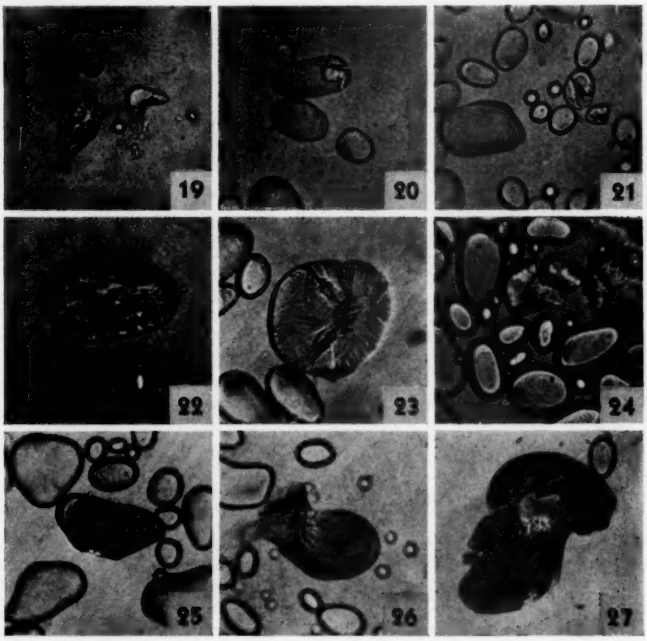

FIG. 6. Miscellaneous potato starch formations. Nos. 19, 20, 21, granules with interior partly digested from within the incomplete coatings. 22, swollen granule without coatings. 23, parted granule as in 24 with point upward. 24, parted granule showing point and pit from which it was withdrawn. 25, granules with new starch formation between them showing loosened coatings with ends bent back. 26, 27, monster granules without coatings.

ments show this apparent tubular construction. It was first noticed under the slow action of diastase from germinated Indian corn. These various fragments are blued with iodine. With very dilute iodine they do not show solid color, but are blued in parts as if made of more than one substance.

It is commonly accepted that enzymes not only break down, but, under other conditions, build up the food reserves. In the young growing potato we see something of how starch granules are formed. Thin, hazy, irregular

aggregates of matter appear as starch on staining slightly with very dilute iodine (10). Various others are becoming gradually more dense (11, 12). Still others are seen partly covered with a thin coating (13, 14). The ragged outline of the irregular aggregates is being subdued and becomes shaped more nearly in the form of a granule. Sometimes irregular portions of considerable size extend from the main portion (12). Others are seen with a heavier coating showing on the surface, the characteristic appearance of a typical granule, but disconnected in places (15, 16, 17).

These incomplete granules can readily be seen scattered among many granules by adding a little dilute iodine. This stains the exposed interior a deep blue while the coating and grains fully coated are little affected. The antecedents of the granules, the appearance of the exposed interior of the incomplete granules, and the results of the action of diastase on them show clearly their lack of organization. When diastase acts on the interior it is eaten away, leaving the coating as a partial shell (19, 20, 21, and many more perfect specimens not here shown). In these imperfect granules we do not obtain the same characteristic segmentations by the continued action of diastase as is found when it acts on mature granules.

The enlargement of granules by deposit of starch aggregates on the surface is frequently seen (18). These become coated as was the original granule and thus become a part of it. Frequently during this process the coating is seen to have been dissolved and the loose ends spread or folded back (25). Numerous large granules show clearly from their outlines that they have been enlarged in this way.

That this general method of starch formation is not confined to the potato is apparent from a similar but less complete study on frosted wheat. Here the kernels are still green from being stopped in the midst of their development. The partially formed granules are somewhat as described for the potato. Photographs of various starches seen in the extensive work of REICHERT (**2**) give evidence of such formation of the starch of other plants. However, the starches used for those several hundred photographs had been separated and purified by washing in water (part I, p. 299). This must necessarily remove a considerable part of the partially formed granules. This volume contains much excellent information concerning the previous literature on starch.

Some unusual photographs are seen in figure 6. In one new potato several large swollen granules were noted. They had thin coats over them. Under the influence of bacterial diastase one was seen gradually to loosen this coat. It then formed a crack and separated into parts leaving the usual long point on the one part and the pit in the other. In various instances the point of a separated granule was directed upward (23). The general uniformity of the outlines of the two parts of a separated granule are shown in

figure 6 (24). The enlarging of a starch granule by formation of an aggregate of starch substance between two granules was occasionally seen (25). Here a part of the coating on each has folded back to permit the inclusion of the new aggregate. In one new potato several uncoated monster granules were seen (26, 27). In dilute iodine these stood out as an intense blue mass. That no coating surrounded them was clearly apparent. This emphasizes the point that in the formation of the starch granule, as in its dissolution, two active agents take part.

In a review of these observations in early April new potatoes, so immature that the skin has peeled freely in shipping, showed many starch granules in the process of formation as described. In several other potatoes, new but more mature, the newly formed granules were rare. This is construed as further confirming the usual sequence in formation of starch as here described.

During the present summer (1937) opportunities have been found for a further study of young and growing specimens of starch-producing substances. These have included certain cereals, tubers of the spring beauty, and other early flowering plants, besides acorns, thus representing widely different families. Some tubers of the Colorado wild potato (*Solanum jamesii*) have also been useful. In the many observations made, there was abundant evidence in all these specimens of conditions similar to those in new potatoes.

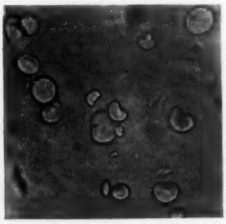

FIG. 7. Starch from young spelt.

In spelt, wheat, barley, and rye, seeds were taken shortly after blossoming and later. At first very minute starch granules were seen. Soon larger crescent shaped granules appeared. From the inner side of many of these a projection was seen. They then showed an outline similar to that of a section through the center of the ordinary cultivated mushroom and parallel to the stem. Gradually there were larger granules and the crescent became filled much as the moon fills during the second quarter. During this stage the inner portion is often seen to be imperfectly coated while the outer curve shows a distinct coating. These forms are seen in figure 7. In many of these seeds, especially in rye, loose aggregates of coagulated starch substance

are seen. These appear as loosely constructed flat discs, such as when coated would form flat or lenticular starch granules like those common to these cereals.

The specimens of Colorado potato tubers examined showed many uncompleted granules and many partly coated aggregates of starch substance. When an active diastase acts upon these imperfect granules the interior is often eaten away leaving portions of overhanging starch coating. This has been seen when using the diastase of malt, molds, bacteria, the saliva, and the pancreas. The ragged overhanging starch coating seen in this way shows a marked contrast to the well-rounded edges of the newly formed coating as seen in slides prepared without the added diastase. These contrasts may be seen in the upper row of figure 6 in comparison with specimens shown in figure 5.

Summary

1. The grains of certain cereals, and parts of some other plants, contain two types of sugar-forming diastase. Both are markedly influenced by changes in temperature and pH but in opposite directions, especially at the higher temperatures of their activity. The one predominates in the flour of wheat, the other in the bran, although both are present in each. The optimum pH value for the former is about 4.5, and for the latter about 5.3 and above.

2. That type of diastase which predominates in wheat bran is widely distributed in the vegetable kingdom. It is most abundant where active vegetation is taking place, and, with the liquefying type, may be called *vegetative diastase.* These two are the types which develop most prominently during germination. That type which predominates in flour is limited in its distribution. It is present in certain substances containing much reserve starch and may be called *reserve diastase.*

3. Closely associated with the vegetative sugar-forming diastase is another which is predominantly liquefying in its properties. Under suitable conditions it will clarify starch paste with great promptness leaving a water-clear solution. Immediately after liquefying, this solution gives a clear blue with iodine and may contain only very small quantities of reducing substance. Under other conditions liquefying diastase removes the outer coating from starch granules and exposes the interior to the action of the other diastases.

4. By a careful study of young growing potatoes, starch granules are seen in various stages of development. There is first a thin amorphous haze which is blued by very dilute iodine. This is followed by successive stages of more dense aggregations. Around each a coating forms. This binds the interior into the completed granule and protects it against solution. Granules are often enlarged by deposits of starch substance at one or more points on the

surface. The coating is extended to cover this and the whole becomes part of the enlarged granule. Evidence of similar starch formation is seen in developing wheat grains and elsewhere.

5. The interior of the granule, as first formed, is amorphous. Later, and apparently only underneath the coating, it becomes organized into series of segments. When the coating is carefully removed as by liquefying diastase, groups of segments, and eventually many segments, are gradually released. These appear as various regular forms, often of geometric outlines; sometimes funnel shaped, sometimes arborescent. Acted on further by diastase they diminish in size, then pass wholly into solution and we find soluble starch, dextrin and/or sugar in their stead.

The author is greatly indebted to Dr. FRITZ LAUN of the Columbus Laboratories for his patience and painstaking efforts in photographing the specimens selected to illustrate points here given.

THE COLUMBUS LABORATORIES
CHICAGO, ILLINOIS

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RESPIRATORY BLOCK IN THE DORMANT SPORES OF *NEUROSPORA TETRASPERMA*

DAVID R. GODDARD AND PAUL E. SMITH
(WITH FIVE FIGURES)

Introduction

SHEAR AND DODGE (**26**) have shown that the ascospores of *Neurospora tetrasperma* are dormant, but that they may be induced to germinate by a short period of heating. This process of heat activation was studied by GODDARD (**7**), who found that heating the dormant spores to temperatures of over 50° C. for a few minutes induced germination two to three hours after returning them to room temperature. Further, he found that the activation is reversible. If the respiration of the activated spores was prevented for several hours by anaerobic conditions or the addition of cyanide, upon returning to conditions favorable for respiration, the spores failed to germinate; that is, they had been de-activated. If the de-activated spores were re-activated by a second heat treatment, they germinated normally. The activation of the spores induced a large increase in the respiratory rate (8 to 40 times), and this high rate of respiration had to continue for two to three hours if germination was to occur. Upon germination, a second increase in the respiratory rate was found, nearly doubling that of the activated spores. Thus, he recognized three phases in the rate of respiration of the ascospores: (1) that of dormant spores, (2) that of activated spores (one-half hour to two hours after the heat treatment), and (3) that of germinating spores (the spores in which germ tubes may be seen under the microscope).

In this paper we have tried to determine what constitutes the respiratory block of dormant ascospores of *Neurospora tetrasperma*, or, conversely, what part of the respiratory mechanism undergoes heat activation. It is to be realized that activation and germination occur readily in distilled water, so that an external substrate is not essential for activation or germination. We may assume concerning the respiratory systems of dormant and activated spores, either (a) that the dormant and activated respiratory mechanisms are qualitatively dissimilar or even independent, and that heat treatment causes a *de novo* appearance of a system inactive in dormant spores, or (b) that the same mechanism is functional in dormant and activated spores, but that in dormant spores the rate is limited by the slowest process in the total reaction chain, and that this slow reaction is greatly accelerated by the heat treatment, allowing the total respiratory chain to proceed at the rate found in activated spores. If the first assumption is correct, the respiratory block is the inactivity of the reaction which prevents the second

respiratory mechanism from functioning, while if the second assumption is correct, the limited activity of a certain reaction constitutes the respiratory block.

We have accepted the WARBURG-KEILIN scheme of cellular respiration as a working theory, and we have summarized this scheme in abbreviated form in diagram I. For reviews of this theory, see KEILIN (**10, 11**) and

DIAGRAM I

PHAEOHEMIN—CYTOCHROME—DEHYDROGENASE THEORY

(1) Reduced phaeohemin + O_2 —Poisoned by CO→ Oxidized phaeohemin

(2) Oxidized phaeohemin + Reduced cytochromes —Poisoned by HCN→ Reduced phaeohemin + Oxidized cytochromes

(3) Oxidized cytochromes + Reduced dehydrogenases → Reduced cytochromes + Oxidized dehydrogenases + H_2O

(4) Reduced substrate + Oxidized dehydrogenase → Oxidized substrate + Reduced dehydrogenase

Oxidized substrate may undergo reaction with a second dehydrogenase, or it may undergo decarboxylation and then oxidation. Flavine enzyme, coenzymes, etc., omitted. Either (3) or (4) inhibited by urethanes.

MELDRUM (**17**). Certain workers have long believed that the first reactions in respiration are identical with the first steps of alcoholic fermentation. The older literature supporting this opinion is reviewed by KOSTYCHEV

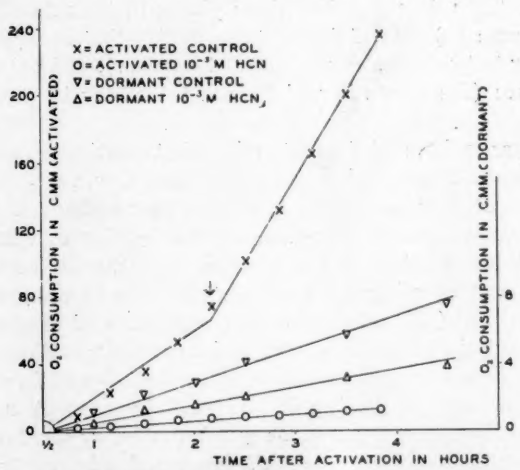
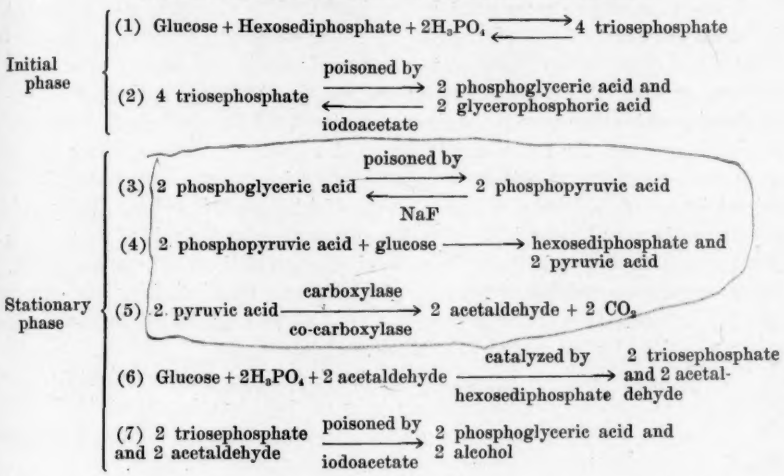


FIG. 1. The effect of 0.001 M. HCN on the respiration of dormant and activated ascospores, 6.48 mg. dry weight of spores per vessel. The arrow indicates the beginning of germination.

(13) and, more recently, by TURNER (32). Since in the work which follows we have had to discuss certain reactions which are usually considered as part of the mechanism of alcoholic fermentation, we have summarized the MEYERHOF-KIESSLING theory of alcoholic fermentation in diagram II. For a review of this theory, see MEYERHOF (19) and MEYERHOF AND KIESSLING (22).

DIAGRAM II

MEYERHOF-KIESSLING SCHEME OF ALCOHOLIC FERMENTATION


Initial phase
(1) Glucose + Hexosediphosphate + 2H3PO4 ⇌ 4 triosephosphate
(2) 4 triosephosphate ⇌ 2 phosphoglyceric acid and 2 glycerophosphoric acid
poisoned by
iodoacetate
Stationary phase
(3) 2 phosphoglyceric acid ⇌ 2 phosphopyruvic acid
poisoned by
NaF
(4) 2 phosphopyruvic acid + glucose → hexosediphosphate and 2 pyruvic acid
(5) 2 pyruvic acid → 2 acetaldehyde + 2 CO2
carboxylase
co-carboxylase
(6) Glucose + 2H3PO4 + 2 acetaldehyde → 2 triosephosphate and 2 acetaldehyde
catalyzed by hexosediphosphate
(7) 2 triosephosphate and 2 acetaldehyde → 2 phosphoglyceric acid and 2 alcohol
poisoned by
iodoacetate

Methods

The *Neurospora tetrasperma* culture was the same strain used in the earlier work (7), and the same method of collecting and separating the ascospores was followed. The cultures were grown at 25° C. for three to four weeks in 500-ml. Blake bottles on 35 to 50 ml. of agar 0.5 per cent. for glucose and Difco malt extract. The spores were stored at constant relative humidity over saturated NH_4Cl/solid NH_4Cl. In the earlier experiments the spores were stored at room temperature, but in the later experiments at about 4° C. Spores stored at room temperature gave fairly uniform results for several weeks; those stored at 4° C. have given very uniform results over several months. In the experiments reported here, the spores from more than four thousand cultures were used, and we wish to thank Miss EUGENIA SHERIDAN who has carried out much of the laborious culture work.

All measurements were made with the FENN (6) volumetric micro-respirometer, at 25° C., unless stated to be at 15° C. This instrument is a

closed system, and is very satisfactory for the low rates of dormant spores. The rate of shaking was 180 complete cycles per minute, through an arc of 1.5 cm. A small spherical glass bead was included in each vessel to enhance stirring. The high density (about 1.18) of the spores caused some shaking difficulties, but in none of these experiments was the rate of shaking limiting. Carbon dioxide was absorbed in KOH insets, unless respiration was measured in the presence of carbon dioxide; in this case, WARBURG's indirect method (5) was used. In the HCN experiments (KREBS, 14), KOH/KCN mixtures were used in the insets. The spores were suspended in M/60 phosphate buffers at a pH of 5.4 or in distilled water. The substrates and poisons were neutralized and made up in the same buffer. The poisons or substrates were added to the vessels at the beginning of an experiment, with other vessels serving as controls. All experiments were run in duplicate, and the experiments reported are only a few of many which gave similar results. The volume of cells used in an experiment was determined by centrifuging to constant volume in hematocrit tubes. Very uniform results may be obtained with *Neurospora spores* by this means. In a few cases, both dry weight and hematocrit determinations were made on the same suspension, and it was found that the volume multiplied by 0.36 equals the dry weight. In most experiments, the $-Q_{O_2}$ was calculated from volume determinations by the use of this factor.

The spores as collected were dormant. Activation was accomplished by heating the spore suspension in a test tube for twenty minutes at 54–56° C. in a water bath, and then cooling to room temperature. Zero was taken as the time at which the spores were removed from the 54° C. bath.

Pure nitrogen for anaerobic experiments was obtained by passing tank nitrogen over freshly reduced copper in an electric furnace. A small piece of yellow phosphorus was used in the inset to absorb any oxygen not swept out of the vessel with nitrogen. Carbon monoxide was prepared by the dehydration of concentrated formic acid with hot sulphuric acid, and was washed with KOH. Gas mixtures were made by volume, and were not checked by analysis.

The hexosediphosphate was a gift from the Winthrop Chemical Company. It was converted to the sodium salt, decolorized with charcoal, and re-precipitated as the calcium salt with alcohol. The calcium salt of the hexosediphosphate was converted to the sodium salt by adding the theoretical amount of sodium oxalate and filtering. The solution of sodium hexosediphosphate was neutralized, when necessary, with HCl. The methyl glyoxal was prepared by M. P. SCHUBERT of the Rockefeller Institute. The pyruvic acid was made by the method of HOWARD and FRASER (9), and was distilled at low pressure.

Results

The respiratory increase on activation and germination

A few typical results are listed in table I. The respiratory rate of activated spores varies from about 8 to 40 times that of the dormant spores,

TABLE I

Respiration of dormant, activated, and germinating spores
Oxygen consumption as $-Q_{O_2}$*

Spore lot	Spore age	Dormant	Activated 1–1.5 hr. after activation	Germination 3–5 hr. after activation
	wk.			
A	3	0.25, 0.27	10.86	19.45
A	9	0.53, 0.59	4.48	9.62
B	3	0.25, 0.24, 0.27	10.36	19.60

* $-Q_{O_2} = \frac{\text{cu.mm. oxygen consumed per hr.}^1}{\text{dry wt. in mg.}}$

[1] The symbols Q_{O_2}, Q_{CO_2}, etc. are by convention negative when the gas is consumed and positive when it is produced.

depending upon the age of the spores. The $-Q_{O_2}$ of dormant spores increases from about 0.25 when first collected to 0.50–0.70 when several weeks older, while the $-Q_{O_2}$ of activated spores decreases from about 10 when first collected to about 5–6 several weeks later. If the spores are stored at 4° C. the values remain practically constant at 0.5 and 5 to 6 respectively for several months. When the spores have been stored for 3 to 4 months at room temperature the $-Q_{O_2}$ values of dormant and activated spores have decreased to about 0.08 and 1 to 2 respectively, and only a few per cent. of the spores germinate after a heat treatment.

Approximately two hours after activation, if respiration has been allowed to proceed normally at 25° C., and four hours, if at 15° C., a second change in respiratory rate occurs, and this corresponds with the first appearance of the germ tubes. The respiratory rate of germinating spores also varies with the age of the spores, but in such a manner that the absolute value is about twice that of the activated rate. The average value from ten experiments with spores of various ages was 1.98 times the activated rate, and the range was from 1.79 to 2.15. The number of spores germinating seemed to be quite independent of the rate of oxygen consumption as long as the $-Q_{O_2}$ for activated spores was 4 to 5 or higher.

Effect of oxygen and carbon dioxide pressure on respiration

It is possible that the permeability of the dormant spores to carbon dioxide or oxygen limits the respiratory rate. If permeability to oxygen

TABLE II

EFFECT OF O_2 AND CO_2 PRESSURE ON DORMANT SPORES
OXYGEN CONSUMPTION AS $-Q_{O_2}$

GAS PRESSURE	OXYGEN*			CO_2†	
	A	B	C	D	E
mm. Hg.					
0	—	—	—	0.33	0.71
37	0.15	—	—	0.30	0.65
75	0.15	—	0.66	0.37	0.66
150	0.17	0.27	0.65	0.30	—
190	—	—	—	—	0.58
300	0.16	0.26	—	—	—
750	—	—	0.77	—	—

* CO_2 pressure = zero.
† O_2 pressure = 150 mm.

were limiting, we would expect an increase in the respiratory rate by raising the partial pressure of oxygen. The data in table II show that raising the oxygen pressure from 37 to 300 mm. has practically no effect on the rate. If the retention of CO_2 by the spores were limiting, it would be expected that increased pressures of CO_2 would decrease the rate. The data in table II show that this is not the case. The results reported in table III

TABLE III

EFFECT OF OXYGEN TENSION ON ACTIVATED AND GERMINATING SPORES
OXYGEN CONSUMPTION AS $-Q_{O_2}$

OXYGEN PRESSURE	A 25° C.		B 25° C.		C 15° C.	
	ACTIVATED	GERMINATING	ACTIVATED	GERMINATING	ACTIVATED	GERMINATING
mm. Hg.						
75	—	—	6.04	6.90	3.07	7.55
150	6.94	10.17	7.91	15.65	3.15	7.03
300	—	—	8.00	16.60	3.23	—
450	8.90	11.40*	—	—	—	—
750	8.92	10.25*	—	—	—	—

* High partial pressures prevent normal germination.

show the effect of oxygen tension on germinating and activated spores. At oxygen tensions below 20 per cent., the oxygen diffusion rate may be limiting the rate of respiration in activated and germinating spores at 25° C. but not at 15° C., where the absolute rate is lower. It seems very improbable that the permeability of the dormant spores to CO_2 or oxygen governs their rate of oxygen consumption.

THE PHAEOHEMIN-CYTOCHROME SYSTEM

The work of WARBURG and KEILIN, which is briefly summarized in diagram I indicates that the cyanide-carbon monoxide sensitive respiration of most cells is catalyzed by the phaeohemin-cytochrome system. From diagram I it can be seen that both cyanide and carbon monoxide poison the phaeohemin enzyme. In an attempt to determine whether the rate of respiration of dormant spores was limited by the activity of the phaeohemin enzyme, we have studied the effect of cyanide and carbon monoxide on the respiration of dormant and activated spores. The results are shown in figures 1 and 2 and in tables IV and V. It is apparent from these results

TABLE IV

PERCENTAGE INHIBITION OF RESPIRATION OF SPORES BY HCN

HCN CONCENTRATION	DORMANT	ACTIVATED	GERMINATING
1.0×10^{-4} Molar	7.2; 5.4; −7.5	41.6; 23.2	55.6; 53.4
1.0×10^{-3} Molar	49.2; 45.3	86.8; 86.8	94.4; 94.1†
1.0×10^{-2} Molar	35.5*	87.5	95.6†

* Result not very reproducible; HCN poisoning is not reversible at 1.0×10^{-2} molar. Lower concentration always reversible. 1.0×10^{-2} molar sometimes stimulates dormant spores slightly, while 1.0×10^{-3} molar always inhibits.

† Controls germinating; HCN prevents poisoned spores from germination.

TABLE V

PERCENTAGE INHIBITION OF RESPIRATION OF SPORES BY CARBON MONOXIDE*

GAS MIXTURE	DORMANT	ACTIVATED	GERMINATING
90% CO†/10% O_2	5.6; −5.0; 7.7; 15.4	53.3; 43.4; 50.2; 31.6; 38.3; 42.3	54.7; 17.1; 26.0; 8.8
95% CO‡/5% O_2	21.4; −6.0	73.3	
Av.	5.7	43.15	23.64

* In the dark.

† Controls 90% N_2; 10% O_2.

‡ Controls 95% N_2; 5% O_2; these results at 15° C.

that the respiration of the activated spores is much more sensitive to these poisons than is the respiration of the dormant spores. Not only are the dormant spores less sensitive to these poisons, but about 50 per cent. of the respiration is entirely resistant to cyanide. A comparison of the absolute rates of respiration of cyanide poisoned dormant and activated spores is of interest. The results from one experiment gave the following: the $-Q_{O_2}$ values for dormant spores and dormant spores plus 10^{-3} and 10^{-2} molar HCN were 0.27, 0.14, and 0.17 respectively. The corresponding values for

activated spores were 10.86, 0.65, and 0.30. These $-Q_{O_2}$ values show that while most of the activated respiration is poisoned by HCN, cyanide does not bring the respiration quite as low as that of cyanide poisoned dormant spores. Cyanide at 10^{-3} and 10^{-2} molar inhibits 93.5 and 97.1 per cent. of the increased respiration due to activation (the increased respiration equals

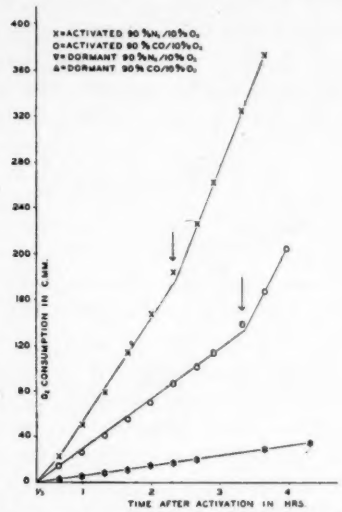

X=ACTIVATED 90 % N2 /10 % O2
O=ACTIVATED 90 % CO/10 % O2
V= DORMANT 90 % N2 /10 % O2
Δ= DORMANT 90 % CO/10 % O2
O2 CONSUMPTION IN C.MM.
TIME AFTER ACTIVATION IN HRS.

FIG. 2. The effect of carbon monoxide on the respiration of dormant and germinating ascospores, 7.95 mg. of spores per vessel in the activated experiment and 14.90 mg. of spores per vessel in the dormant experiment. The arrows indicate the beginning of germination.

the activated respiration minus the dormant respiration). The cyanide resistant respiration may be catalyzed by WARBURG'S flavine enzyme since it is known (**30**) that the enzyme is not cyanide sensitive. However, it is clear that 93 to 97 per cent. of the increased respiration due to activation is cyanide sensitive, so that the activity of the flavine enzyme (if it is present) cannot account for the increased respiration on activation. The carbon monoxide results show that the dormant respiration is almost completely carbon monoxide resistant, while the activated respiration is definitely carbon monoxide sensitive.

If the rate of respiration of the dormant spores is limited by the concentration of active phaeohemin, the dormant respiration should be as sensitive to cyanide and carbon monoxide as is the respiration of the activated cells. However, if the concentration of the phaeohemin is as high in the dormant spores as in the activated spores, only 1/8 to 1/40 of the enzyme is essential to maintain the observed rate, and poisoning a large percentage

of the enzyme with carbon monoxide or cyanide will have little or no effect on the dormant rate, while a large inhibition of the activated rate may be expected. This is what has been found experimentally. Cyanide at 10^{-4} molar should combine with about 50 per cent. of the phaeohemin, but it has no effect on the dormant respiratory rate. Higher concentrations, as 10^{-3} molar, will combine with 95 per cent. or more of the enzyme. The affinity of carbon monoxide for phaeohemin is much less than is that of cyanide, and it may be impossible, at pressures of atmospheric or less, to tie up sufficient phaeohemin in dormant cells to produce an appreciable inhibition. Following this line of argument, we have interpreted our cyanide and carbon monoxide results to mean that the respiratory rate of the dormant spores is not determined by phaeohemin activity.

In the WARBURG-KEILIN scheme, cytochrome is believed to act as a carrier between phaeohemin and the dehydrogenases. No specific cytochrome poison is known. Cytochrome activity may be followed spectroscopically in some cases, but the black spore pigment has made that impossible in this work. One method of attack on phaeohemin-cytochrome systems is the one introduced by BARRON (**2**), and used by many workers, of "short circuiting" both the phaeohemin oxidase and cytochrome by the addition of autoxidizable dyes (such as methylene blue, pyocyanine, and thionine) to the cell suspensions. These dyes are reduced by dehydrogenase-substrate systems and re-oxidized directly by atmospheric oxygen, and therefore these dyes may replace both oxidase and cytochrome. Another dye which RUNNSTROM (**25**) has used in paraphenylenediamine. This dye is not autoxidizable. It is reduced by the dehydrogenase-substrate systems, and its oxidation is catalyzed by phaeohemin. This dye will supplement cytochrome, but not phaeohemin. We have added such dyes to dormant spores, and no significant change in the respiratory rate has been detected, as is seen in table VI. These results may be interpreted to mean that the respiratory block is not

TABLE VI

EFFECT OF DYES ON DORMANT SPORES

OXYGEN CONSUMPTION AS Q_{O_2}

DYE	CONTROL	EXPERIMENTAL
Methylene blue 0.01%	0.23	0.31
Pyocyanine M/5000	0.31	0.24
Pyocyanine M/10,000	0.31	0.31
Paraphenylenediamine M/1000	0.21	0.28*
Paraphenylenediamine 10 mg. per vessel	0.37	0.46†

* Not corrected for autoxidation of the dye.

† Corrected for autoxidation of the dye.

in the phaeohemin-cytochrome system, or that the spores are impermeable to the dye, and that no conclusions can be drawn from these experiments.

To prove definitely that the oxidase is phaeohemin, light reversal of carbon monoxide poisoning should be demonstrated. Attempts to obtain light reversal of carbon monoxide poisoning were largely unsuccessful, probably because of the black pigment in the spore walls. The results all show slightly less inhibition by carbon monoxide in the light (7 cm. from 100-watt Mazda lamps) than in the dark, but the differences were hardly larger than the experimental error and are not reported here.

Paraphenylenediamine may be used to measure the activity of phaeohemin, by the method of KEILIN (**10**). In this method, a large amount of dye (10 to 23 mg.) is added to the cell suspension, and the oxygen used in oxidation of the dye is measured. Proper controls are used to correct for the autoxidation of the dye and for the cellular respiration (or this may be poisoned with urethane, etc.). The rate of the catalyzed oxidation of the dye is a measure of phaeohemin activity. Such an experiment was tried with dormant spores with negative results. This may mean that there is no phaeohemin activity (which would conflict with our CO and HCN results), or, more likely, that the cells are impermeable to the dye.

DEHYDROGENASE-SUBSTRATE SYSTEMS

It seems to be clearly established from the work of KEILIN (**10**) and others that the respiratory substrates do not react directly with oxidized cytochrome, but that the substrates first react with intermediary enzymes, the dehydrogenases of THUNBERG (**31**). The usual method of determining dehydrogenase activity is the methylene blue technic of THUNBERG. An attempt to use this method with *Neurospora* spores failed because the black spores removed all of the dye from solution, and the spore color made it impossible to determine whether the dye was reduced or not. (An attempt to determine this spectroscopically also failed.) The dye may not be extracted by centrifuging with water, though it can be extracted with acetone.

We know no truly specific dehydrogenase poison. Though SVENSSON (**29**) has shown that urethane will poison dehydrogenases, other enzymes are also poisoned by urethane. We have studied the effect of ethyl urethane on the respiration of dormant and activated spores, and a typical experiment is shown in figure 3. A higher concentration of urethane (5 per cent.) caused at first a marked decrease in respiration of dormant spores, followed by stimulation. The stimulation is probably associated with irreversible injury. If the percentage inhibition of respiration by 2.5 per cent. urethane is calculated from the data in figure 3 for dormant, activated, and germinating spores, the following results are obtained: 48, 19, and 62 per cent.

respectively. The greater sensitivity of the dormant than of the activated spores to urethane might be interpreted to mean that the dehydrogenase systems are limiting the dormant rate. However, we have not been able to obtain more direct evidence on this point. The results reported later on carboxylase activity seem to be sufficient to explain the respiratory increase following activation, without the necessity of a change in dehydrogenase

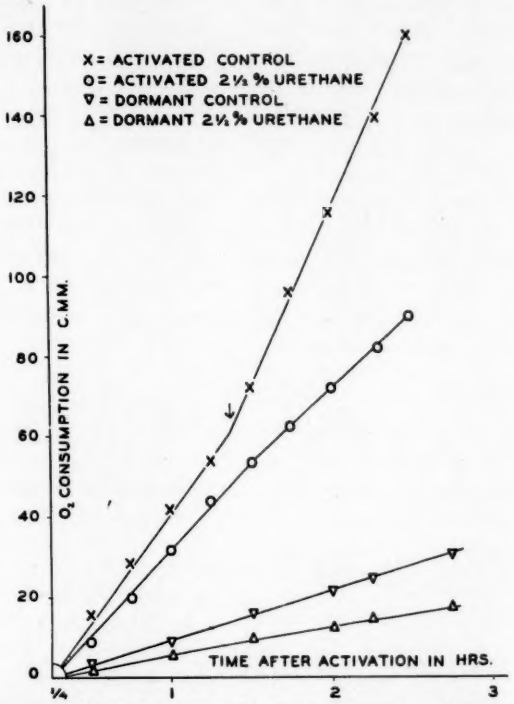


FIG. 3. The effect of 2.5 per cent. ethyl urethane on the respiration of dormant and activated spores. The arrow indicates germination; the poisoned activated spores did not germinate. 10.1 mg. of spores per vessel in the activated experiment, and 40.4 mg. of spores per vessel in the dormant experiment.

systems. The low sensitivity of activated spores to urethane remains without explanation.

Since spores may undergo activation and germination in distilled water, it is clear that the amount of stored food is not limiting the respiration of dormant spores. However, it is possible that though sufficient stored substrate is present, the rate at which it undergoes hydrolysis, phosphorylation, or other anaerobic reactions may be limiting the rate of respiration. If this

were the case, the addition of the proper intermediate substrate should cause a marked increase in the respiratory rate of dormant spores. It is seen from the results in table VII that the only substrates which have any

TABLE VII

EFFECTS OF SUBSTRATES ON OXYGEN CONSUMPTION OF DORMANT SPORES
VALUES GIVEN AS $-Q_{O_2}$

SUBSTRATE	CONCENTRATION	SUBSTRATE RESPIRATION	CONTROL	PERCENTAGE STIMULATION
Glucose	M/50	0.345	0.355	-2.8
Sodium hexosediphosphate	M/50	0.344	0.355	-3.2
Acetaldehyde	M/50	0.707	0.355	99.2
Above three together	M/50	0.737	0.355	107.6
Ethyl alcohol	M/20	1.150	0.529	117.5
Acetaldehyde	M/50	1.152	0.529	117.8*
Alcohol plus acetaldehyde	M/20 + M/50	1.178	0.529	122.8
Methyl glyoxal	M/50	0.420	0.357	15.0
Sodium succinate	M/20	0.550	0.528	4.16
Pyruvic acid	M/20	0.296	0.273	8.40
Sodium glycerophosphate	M/20	0.286	0.273	4.78
Sodium acetate	M/40	0.301	0.303	-0.6

* Stimulations as high as 228% have been obtained at M/20, and 297% at M/50 acetaldehyde.

appreciable effect are acetaldehyde and ethyl alcohol. The increase caused by these compounds is not additive. Though the stimulation in respiration caused by these compounds is marked, it is far short of the stimulation induced by heat treatment. The possible significance of acetaldehyde and alcohol stimulation will be discussed later. It is seen from diagram II that alcoholic fermentation depends upon the presence of glucose, hexosediphosphate, and acetaldehyde. In an experiment these substances were added to one vessel containing dormant spores, and each substance independently to other spores, and the oxygen consumption followed. The increased oxygen consumption was no larger than was obtained from acetaldehyde alone, as may be seen from table VII. The failure of the added substrates to bring about respiratory stimulation may be due to impermeability of the spores or because the proper substrate or combination of substrates has not been found. The results obtained above with added substrates have not ruled out the possibility that the respiratory block is located in the anaerobic reactions which act on glycogen and which form the actual respiratory substrate.

Action of sodium fluoride, iodoacetate, and iodoacetamide on oxygen consumption

Blackman's theory of plant respiration postulates that the first step in aerobic respiration is the conversion of carbohydrates to trioses or triose-phosphates in reactions essentially similar to those of alcoholic fermentation. This theory has recently been reviewed by Turner (**32**). One approach to the possible location of the respiratory block between glycogen and the actual respiratory substrate is to study the effect of certain poisons, known to inhibit alcoholic fermentation and muscle glycolysis, on oxygen consumption. In this section we report some results on the action of sodium fluoride, iodoacetate, and iodoacetamide on oxygen consumption. We cannot say definitely what steps of the respiratory mechanism are inhibited by these poisons, but it is possible that the same reactions which are poisoned in anaerobic metabolism are also inhibited in respiration, even though the concentrations required to inhibit respiration are higher than those which inhibit fermentation. The question has been discussed at some length with special attention to iodoacetate by Turner (**32**).

Sodium fluoride poisons alcoholic fermentation and has been shown by Lohmann and Meyerhof (**15**) to poison the breakdown of phosphoglyceric acid to pyruvic acid and H_3PO_4 (see diagram II). It is not claimed that this is the only reaction poisoned by fluoride. Lundsgaard (**16**) has shown that iodoacetate poisons muscle glycolysis and alcoholic fermentation, and it has been shown by Meyerhof and Kiessling (**20, 21**) that it does not inhibit the breakdown of phosphoglyceric acid to acetaldehyde and CO_2, but that it does inhibit reactions 2 and 7 of diagram II. It is possible that other reactions may also be poisoned by iodoacetate. It is well known that iodoacetate combines with –SH groups of glutathione (**4, 23**) and proteins (**8, 24**), but the work of Smythe (**27**) indicates that its inhibition of fermentation is not due to destruction of –SH groups. Goddard (**7**) has shown that iodoacetamide inhibits respiration of *Neurospora* spores, Smythe (**27**) that it inhibits alcoholic fermentation, and Stannard (**28**) that it inhibits muscle glycolysis. The mechanism of the action of this compound is unknown, though it is established that it reacts rapidly with –SH groups (**8, 27**). The results obtained with these three poisons on the spores are shown in table VIII. The results of using iodoacetate and iodoacetamide on activated and germinating spores are recalculated from an earlier paper (**7**).

The great sensitivity of all phases of the respiration to sodium fluoride is the most interesting result from these experiments. If the respiration is over pyruvic acid, this result is to be expected, but this does not explain the inhibition of the respiration of ethyl alcohol and acetaldehyde by fluoride. It is possible that neither of these compounds is directly undergoing oxidation, but they may be entering into dismutation reactions similar to

TABLE VIII

EFFECTS OF POISONS AND SUBSTRATES ON OXYGEN CONSUMPTION OF SPORES
VALUES AS $-Q_{O_2}$

POISON	SUBSTRATE	DORMANT			ACTIVATED			GERMINATING		
		CONTROL	EXPERIMENTAL	PERCENTAGE INHIBITION	CONTROL	EXPERIMENTAL	PERCENTAGE INHIBITION	CONTROL	EXPERIMENTAL	PERCENTAGE* INHIBITION
NaF M/10	None	0.32	0.067	81.5	5.68	1.61	64.7	15.75	0.76	95.2
NaF M/10	Acetal. M/50	0.34	0.19	73.8†	—	—	—	—	—	—
None	" "	0.34	0.70	—	7.31	7.71		14.4	16.7	—
NaF M/10	Alcohol M/20	0.54	0.15	78.6‡	—	—	—	—	—	—
None	" "	0.54	0.89	—	—	—	—	—	—	—
Iodoacetate M/200	———	0.47	0.46	2.0	4.07	4.11	−1.0	7.36	3.46	53
Iodoacetate M/100	———	0.47	0.43	8.5	—	—	—	—	—	—
Iodoacetamide M/200	———	0.50	0.30	40.0	4.07	3.70	8.2	7.36	2.43	67

* In no case did the poisoned spores germinate. These figures are obtained by comparing germinating controls with poisoned cells, at the same time periods after activation.

† Inhibition calculated against the rate with acetaldehyde from the experiment below.

‡ Inhibition calculated against the rate with alcohol from the experiment below.

reactions 6 and 7 of diagram II. This would seem probable enough for acetaldehyde but much less probable for alcohol, unless it is first converted to acetaldehyde.

The respiration of the germinating spores is sensitive to iodoacetate (pH 5.4) and the spores are rapidly killed by the poison. The oxygen consumption of dormant and activated spores is unaffected by iodoacetate and it is probable that it does not penetrate the cells. If dormant or activated spores stand for an hour in iodoacetate (0.005 M) and are then washed on the centrifuge, they germinate normally. The respiration of dormant and germinating spores is poisoned by iodoacetamide, while the activated spores are unaffected (see table IX). This cannot be due to failure to penetrate the

TABLE IX

ANAEROBIC CO_2 PRODUCTION OF ACTIVATED SPORES IN THE PRESENCE OF SODIUM FLUORIDE, IODOACETAMIDE, AND ETHLY URETHANE

MEDIUM	$Q_{CO_2}^{N_2}$*	PERCENTAGE INHIBITION
Control	5.30	
NaF M/10	1.44	73.0
Control	7.18	
Iodoacetamide M/200	5.82	19.0
Iodoacetamide M/1000	7.07	1.5
Control	8.13	
Ethyl urethane 2½ per cent.	7.35	10.0

* $Q_{CO_2}^{N_2} = \frac{\text{cu. mm. } CO_2 \text{ per hour}}{\text{dry wt. mg.}}$ (under anaerobic conditions).

$Q_{CO_2}^{N_2}$ and percentage inhibition calculated on the linear part of the curve.

activated spores, for if activated spores are exposed to iodoacetamide and then washed in the centrifuge, the respiration proceeds normally until the time when the controls germinate; then the respiration of the poisoned washed cells falls off and no germ tubes are formed. There seems to be a real difference in the respiratory mechanisms of dormant and germinating spores on the one hand and of activated spores on the other, with reference to iodoacetamide poisoning. No explanation of this effect seems possible at this time.

ANAEROBIC CO_2 AND CARBOXYLASE ACTIVATION

The marked inhibition of the oxygen consumption by sodium fluoride suggested that reactions similar to those occurring in alcoholic fermentation might be part of the respiratory scheme. The best approach to such reactions seemed to be through a study of the anaerobic CO_2 production. Re-

sults typical of many experiments are shown in fig. 4. It may be seen from the figure that no measurable anaerobic CO_2 is liberated by the dormant spores. Even when 40 mg. of dry weight of dormant spores were used, no CO_2 evolution was detected which was greater than the experimental error ($\pm$ 1.0 cu.mm. per hour). The activated spores evolved CO_2 anaerobically quite rapidly as may be seen in fig. 4 and table IX. The rate of anaerobic CO_2 production by the activated spores is linear for a short period after activation and gradually falls to zero at about three hours after the heat

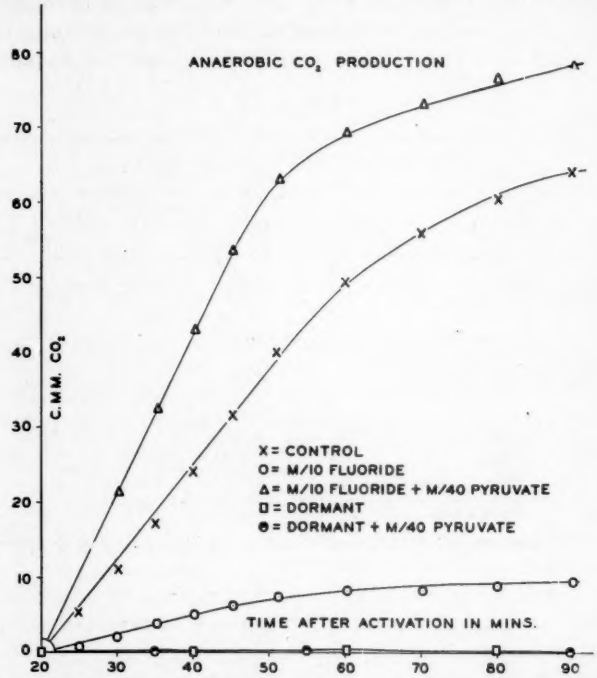


FIG. 4. Anaerobic CO_2 production of dormant and activated spores. The effect of 0.1 M NaF on inhibition of CO_2 production of activated spores. The effect of pyruvate and NaF together on activated spores and the failure of pyruvate to affect dormant spores. 19.5 mg. of spores per vessel.

treatment. The rate of anaerobic CO_2 formation by activated spores during the linear phase is about the same as the rate of oxygen consumption. Compare tables I and IX. Though the dormant spores liberate no CO_2 under anaerobic conditions, it was expected that they would produce CO_2 aerobically. Aerobic CO_2 production was measured by WARBURG'S indirect method (see DIXON, 5) with the suspension in equilibrium with an atmos-

phere of 5 per cent. CO_2 and 95 per cent. oxygen. Only a few experiments were made, and a typical one gave a $Q_{CO_2}^{O_2}$ of 0.59 and a respiratory quotient of 0.85 $\left(Q_{CO_2}^{O_2} = \frac{\text{cu.mm. } CO_2 \text{ evolved per hr., in the presence of } O_2}{\text{mg. dry weight}} \right)$.

Formation of anaerobic CO_2 is often accompanied by formation of alcohol or organic acids. No attempt has been made to determine whether the activated spores produce alcohol under anaerobic conditions. Acid formation may be determined manometrically by the displacement of CO_2 from a bicarbonate buffer according to the following equation:

$$HA + NaHCO_3 \rightarrow NaA + CO_2 + H_2O$$

Under anaerobic conditions the difference in CO_2 liberated from bicarbonate buffer and from phosphate buffer is a measure of the acid formed. We carried out such an experiment with activated spores in M/60 phosphate buffer with pH 5.4 and in 0.0025 M $NaHCO_3$ in equilibrium with 5 per cent. CO_2, with pH 7.4. The average from two vessels was 52.8 c.mm. in one hour in phosphate buffer, and 52.5 cu.mm. in bicarbonate buffer. We may be sure that under anaerobic conditions the activated spores do not form any acid.

If the block in anaerobic CO_2 production of the dormant spores was anterior to pyruvic acid formation (see diagram II), it was to be expected that the addition of pyruvic acid to dormant spores would induce anaerobic CO_2 production. The pyruvic acid solution used was half neutralized, so that the $pH = P_{ka} = 2.5$, and one half of the acid was present in undissociated form. Under these conditions (and in unneutralized pyruvic acid or in sodium pyruvate solutions), no anaerobic CO_2 could be detected from dormant spores (see fig. 4). This failure of dormant spores to produce CO_2 from pyruvate suggested that there was no active carboxylase in dormant spores.

Experiments on the effect of three poisons on anaerobic CO_2 production are shown in table IX. In each case the $Q_{CO_2}^{N_2}$ and the percentage inhibition were calculated on the linear part of the curve (fig. 4). The results with NaF are striking, and are in agreement with the MEYERHOF-KIESSLING theory of alcoholic fermentation. This experiment and the one which follows make it clear that the anaerobic CO_2 is not merely the physical release of bound CO_2 due to heating, nor to the escape of previously formed CO_2 due to changes in cell permeability. From the previous discussion, it seems quite clear that iodoacetamide penetrates the spores, and it was surprising that it had such a small effect on anaerobic CO_2 production. The small effect of ethyl urethane is not surprising. This experiment was included

for comparison of its effect on oxygen consumption and anaerobic CO_2 production.

The fact that dormant spores liberate no anaerobic CO_2 even when pyruvic acid is added, and the ready production of CO_2 by activated cells suggests that carboxylase is absent or inactive in dormant spores, and that this enzyme is activated on heat treatment. If this is true, we should be able to demonstrate carboxylase activity by restoring the ability of NaF-poisoned activated spores to produce anaerobic CO_2 by the addition of pyruvic acid. NaF is known to poison anaerobic CO_2 formation from glucose (see diagram II), but not from pyruvic acid, since it does not poison carboxylase. The increased CO_2 production on the addition of pyruvic acid over that of NaF-poisoned cells should be an approximate measure of carboxylase activity. The results from one such experiment are shown in figure 4. It is clear that pyruvic acid raises the amount of CO_2 production above that of the unpoisoned controls; therefore, in the controls the rate of CO_2 production is not limited by carboxylase concentration, but by the rate of pyruvic acid formation. We interpret the failure of pyruvic acid to induce anaerobic CO_2 production in dormant spores and its pronounced effect on NaF-poisoned activated spores as definite evidence that the enzyme carboxylase is activated (formed?) on heat treatment of the spores.

It will be recalled that heat activation as it affects germination and increased respiration is reversible; that is, under anaerobic conditions activated spores return to secondary dormancy. These de-activated spores respond to a second heat treatment in the usual manner. The falling off in the rate of anaerobic CO_2 production, with or without added pyruvic acid, is surprisingly similar to the de-activation of the germination mechanism under the same conditions. An experiment was undertaken to determine whether carboxylase activation was likewise reversible. After the activation of the spores, a sample was set aside in a hanging drop (in air) for a germination test. The spores were placed in four vessels, and anaerobic CO_2 production measured. After the rate had fallen off (170 min.), two vessels were removed from the bath and spore samples were taken for germination tests. (The samples were too small to affect the subsequent readings.) The two vessels were placed for twenty minutes in a thermostat bath at 55° C. for re-activation. After the re-activation, samples were again removed for germination tests, the vessels replaced in the bath, anaerobic conditions established, and the CO_2 production again measured. During this period, readings were continued on the other two vessels. The experiment was continued for six hours after the first activation, and by this time the rate of CO_2 production had fallen approximately to zero in all vessels. The experimental results are shown graphically in figure 5.

Examination of figure 5 shows that the activation, de-activation, and

re-activation of the spores as concerns anaerobic CO_2 production (and presumably carboxylase activity) is closely parallel to the reversible activation-de-activation of the germination mechanism.

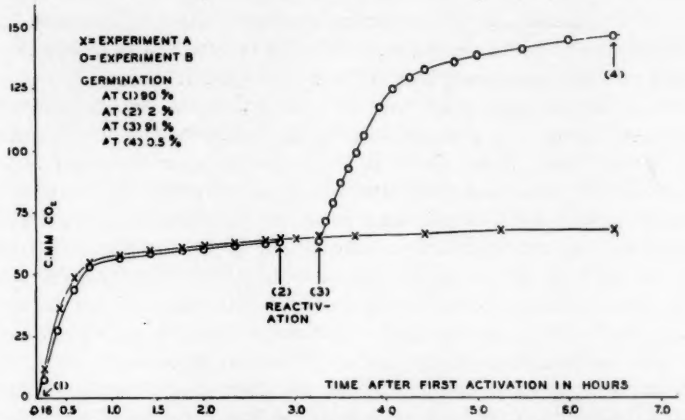


FIG. 5. The effect of a second heat treatment on the anaerobic CO_2 production. In experiment A, the readings were uninterrupted. In experiment B, the vessels were removed at arrow no. 2, re-heated for twenty minutes at 55° C., allowed ten minutes for temperature equilibrium, and readings commenced at arrow no. 3. Germination tests were made by removing the spores to air and counting several hours later, at each point indicated by an arrow.

Discussion

It is possible that the respiratory rate of dormant spores is limited by cell permeability and that activation brings about a reversible change in the cell surface. The authors do not believe that this explanation is the correct one for the following reasons:

(1) The respiratory rate of the dormant spores is independent of the partial pressure of oxygen over the range of 37 to 300 mm. of mercury. This result indicates that the rate of oxygen entrance is not limiting the respiratory rate.

(2) The rate of oxygen consumption of dormant spores is independent of the partial pressure of carbon dioxide over the range of 0 to 190 mm. of mercury. This indicates that outward diffusion of carbon dioxide is not limiting the rate of oxygen consumption.

(3) The spores are not completely impermeable to carbon dioxide, for it is formed under aerobic conditions. The complete failure of dormant spores to liberate carbon dioxide anaerobically cannot be explained on a basis of permeability to carbon dioxide, for the anaerobic CO_2 production would be as high as the aerobic CO_2 production.

(4) The production of anaerobic CO_2 by activated spores might be interpreted as the release of preformed CO_2 due to a change in cell surface, but this seems improbable, for the anaerobic CO_2 production is poisoned by NaF and the NaF poisoning is overcome by pyruvic acid.

(5) Ethyl alcohol and acetaldehyde cause a marked increase in the respiratory rate of dormant spores. This does not seem to be consistent with the view that the respiratory rate is limited by the cell surface.

The much greater sensitivity of the activated spores to carbon monoxide and to cyanide suggests that the activity of phaeohemin is not limiting the rate of respiration. The evidence in this paper does not definitely establish the fact that the oxidase is phaeohemin (indophenol oxidase). To prove this point, light reversal of CO poisoning would be necessary, and this is difficult to obtain with spores with black walls. Proof of phaeohemin might be obtained by the catalysis of paraphenylenediamine, but the spores appear to be impermeable to this dye. This dye could be tried on ground cells, but the authors decided to limit this paper to results obtained with intact living cells. The limited permeability of the spores has made it difficult to obtain any definite information concerning the dehydrogenase systems. It is possible that information may be gained on this point from ground cells, but how useful such information would be in attempting to interpret the changes which occur on activation is open to question. No information has been obtained on cytochrome, for the black spore wall has prevented spectroscopic determinations, and the impermeability of the spores has prevented the use of artificial carriers, such as paraphenylenediamine. It is possible that a portion of the dormant respiration is catalyzed by WARBURG'S flavine enzyme. However, the activated respiration is probably not catalyzed by this enzyme, since the respiration catalyzed by the flavine enzyme (**30**) is cyanide- and carbon monoxide-stable.

Since increased respiration and normal germination are obtained when the ascospores are activated in distilled water, the respiratory block of the dormant spores cannot be caused by absence of stored food. However, it is possible that the reactions converting the stored food into the substrate actually respired may be limiting the respiratory rate. The only added substrates, out of a long list tried, which produced an appreciable increase in the rate of oxygen consumption of dormant spores were acetaldehyde and ethyl alcohol. These substrates stimulated the rate from 100 to 300 per cent., while the difference between dormant and activated respiratory rates is of the order of 1000 to 4000 per cent. The significance of the increase brought about by acetaldehyde or alcohol is not clear. The acetaldehyde and ethyl alcohol respiration is NaF sensitive. This is rather surprising, because NaF does not poison most dehydrogenase systems, nor the phaeohemin-cytochrome system. However, an old result of MEYERHOF'S (**18**) indi-

cates that succinic dehydrogenase is poisoned by NaF. Acetaldehyde may be oxidized by the xanthine oxidase. Ethyl alcohol will undergo a coupled oxidation by xanthine oxidase when hydrogen peroxide and catalase are present (**12**).

The failure of dormant spores to produce CO_2 anaerobically in the presence of pyruvic acid, and the fact that pyruvic acid restores the anaerobic CO_2 production to NaF-poisoned activated spores, seems clearly to establish the fact that carboxylase is inactive in dormant spores and active in heat-treated spores. The reversible activation of the system producing anaerobic CO_2 on heat treatment parallels almost completely the effect of this heat treatment on germination and respiration. The absolute rate of anaerobic CO_2 production during the linear portion of the curve is of the same order as the rate of oxygen consumption. It is difficult to determine whether or not the respiratory block is the absence of active carboxylase, but the agreement between carboxylase activity and the respiration and germination of the spores seems too close to be fortuitous, particularly so when it is recalled that activation and de-activation for one component corresponds with activation and de-activation for the other two. Taken all together, it seems probable that carboxylase activation by heat treatment of the spores leads to a marked increase in the respiratory rate and to germination. It is, of course, possible that other enzymes are similarly activated on heat treatment, and that the inactivity of one or several of these enzymes constitutes the respiratory block.

Since there seems to be no carboxylase activity in dormant spores, but a measurable respiratory rate, it seems clear that the respiratory system of dormant spores is not a system involving carboxylase. From the discussion presented it appears that the respiratory mechanism of the activated spores includes carboxylase as one essential step. If this interpretation is correct, we are forced to the conclusion that there are two qualitatively different respiratory mechanisms, namely, the dormant mechanism of which carboxylase is not a part, and the mechanism which comes into play after the heat treatment, which so closely parallels carboxylase activity that we may assume that carboxylase is one step in the system. The phaeohemin-cytochrome system and other enzymes may be common to the two systems.

If we accept the interpretation that carboxylase is an essential part of the respiratory mechanism of activated spores, then it seems clear that in this system anaerobic processes similar to those in alcoholic fermentation are a part of the respiratory mechanism. It is difficult to understand how carboxylase functions in the respiratory mechanism. It cannot be merely the formation of acetaldehyde, for though the addition of this substance causes a respiratory stimulation, it is far short of the maximum rate. Nor can it be merely the accumulation of pyruvic acid that limits the rate, for the addi-

tion of pyruvic acid does not inhibit the respiration of activated spores. AUHAGEN (1) has shown that a co-carboxylase is essential for carboxylase activity; activation might be the formation of co-carboxylase. If co-carboxylase were identical with a co-enzyme of respiration, the parallelism between carboxylase activity and respiration would be explained, without the necessity of assuming that carboxylase was part of the respiratory mechanism. But AUHAGEN has shown that co-carboxylase is not identical with co-zymase, nor is it found in muscle, which lacks carboxylase but which contains a respiratory co-enzyme. To the authors' knowledge, WARBURG's respiratory co-enzyme from horse blood has not been tried with carboxylase.

The zymase complex including carboxylase is widely distributed in the tissues of higher plants, which are never known to produce alcohol in nature. KOSTYCHEV (13) and BLACKMAN (3) assume that the function of the zymase system is to convert sugars into the respiratory substrates. The BLACKMAN theory assumes that these substrates are trioses. It is possible that carboxylase functions in the respiratory scheme in higher plants, converting three carbon atom keto acids into two carbon atom compounds which are then respired.

Summary

1. An attempt has been made to locate the respiratory block in dormant *Neurospora tetrasperma* ascospores.

2. The respiratory rates at various partial pressures of oxygen and carbon dioxide indicate that permeability of the spores to gases is not limiting.

3. Studies on the cyanide and carbon monoxide sensitivity of dormant and activated spores indicate that phaeohemin (indophenol oxidase) activity is not limiting.

4. The dormant spores produce no CO_2 anaerobically, while the activated spores have a $Q_{CO_2}^{N_2}$ of 5 to 8, though the rate falls to zero after 3 to 4 hours.

5. It is suggested that no active carboxylase is present in dormant spores, but that carboxylase is reversibly activated on heat treatment. The activation-de-activation of carboxylase parallels the effect of activation and de-activation on respiration and germination.

6. The results in this paper are interpreted to mean that two qualitatively different respiratory systems are present, namely, the dormant system, which functions in the absence of carboxylase, and a second system, active in heat treated spores, which passes over the enzyme carboxylase. The respiratory block is then the inactivity of the enzyme carboxylase.

7. These experiments indicate that reactions similar to those of alcoholic fermentation are a part of the respiratory mechanism in *Neurospora.*

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AMMONIUM NUTRITION AND METABOLISM OF ETIOLATED SEEDLINGS

LELAND BURKHART
(WITH ELEVEN FIGURES)

Introduction

The nitrogen metabolism of germinating seeds and young seedlings concerns itself chiefly with the decomposition of already existing proteins stored in the kernels, followed by the translocation and regeneration of the simpler substances into proteins in the meristematic and actively growing regions of the developing plant body. Studies of etiolated seedlings seem suitable for the purpose of obtaining information concerning the intermediate products of protein metabolism, since protein decomposition is favored while its regeneration is hindered in the absence of light because of the depletion of available carbohydrates.

The rôle of available carbohydrates in altering the nitrogen relations of etiolated seedlings supplied with ammonium salts has been emphasized by PRIANISCHNIKOW and his co-workers (**29, 30, 31, 33, 34**); however, no attempt was made to follow the changes in the carbohydrate relations quantitatively. The seedlings, grown in water cultures for a period of 10 days in the dark, were classified into three groups:

I. The cereal type (barley, maize), and the pumpkin when supplied with ammonium salts, showed increases in total nitrogen and amides, but no increase in ammonia.

II. The starchy legumes (pea, vetch) increased in total nitrogen and amides only when ammonium salts were accompanied by calcium carbonate.

III. The yellow lupine, high in protein, showed serious disturbances in the synthetic reactions, manifested by accumulation of ammonia and decrease of asparagine. Addition of calcium carbonate failed to restore the normal course of nitrogen metabolism.

PRIANISCHNIKOW deserves considerable credit for his choice of seeds with such a wide range of food reserves, which seems to have been a determinative factor in causing the different responses of the various seedlings to ammonium nutrition as reported by him. The seeds used differ greatly with respect to the relative amounts of nitrogen-free and nitrogen-containing reserves. The respective ratios used as an index to these differences are as follows: cereals, (6:1); starchy legumes, (2:1); and yellow lupine, (0.6:1).

In order to obtain additional information as to the rôle of carbohydrate reserves as affecting the response of seedlings to ammonium salts, so-called "artificial types" were employed in modifications of experiments previously mentioned. SMIRNOW (**54**) compared the nitrogen metabolism of etiolated

seedlings of high carbohydrate reserve (barley) and low carbohydrate reserve (yellow lupine), which were grown in sterile culture solutions. In the early stages of growth, ammonia was readily absorbed by the barley seedlings, followed by an increase in asparagine. In the later stages, when the carbohydrate reserves were exhausted (21 days), ammonia accumulated in the plants and asparagine decreased (an artificial lupine type physiologically). The presence of calcium salts promoted asparagine formation, but the processes ended sooner, as carbohydrates were more readily exhausted. The absorption of ammonium by lupine seedlings was dependent upon the presence of sugar in the medium. The addition of glucose along with the ammonium salts resulted in an "artificial physiological cereal type."

Although the results of these investigations have often been referred to in the literature, little attempt has been made to confirm these findings. The writer has previously criticized the methods employed by these workers (**2**). In the light of modern principles of mineral nutrition, the use of unbalanced nutrient solutions and distilled water as a control in growing their plants seems very undesirable. Calcium must play a more prominent rôle in altering the response of seedlings to ammonium salts than was realized by PRIANISCHNIKOW when he originally planned his experiments (**32, 33, 38, 60**). Not having separated the principal storage organs from the remainder of the seedlings, no basis was provided upon which to determine the extent of protein regeneration, all of which should be of interpretative value. The ammonia determinations are of questionable value, since the seedlings were dried prior to analysis.

With the adaptation of improved methods, this investigation involves a further study of the effects of ammonium nutrition on the carbohydrate and nitrogen relations of etiolated seedlings as altered by the type and amount of food reserves in the seed. An attempt is made to throw additional light upon certain fundamental questions: What determines the rate and amount of ammonium absorption by seedlings? How and to what extent is the ammonium utilized? What conditions are associated with so-called "ammonium injury"?

Materials and cultural methods

CHOICE AND COMPOSITION OF SEEDS

In this study it seemed desirable to employ seeds of species representing a wide range in ratios of non-nitrogenous reserves to nitrogenous reserves. Seeds were chosen in which the cotyledons served as the storage organs, thus minimizing morphological variations. Seeds of one species, with the desired range of food reserves in the various varieties, would constitute more nearly an ideal choice; however, such are not available. Variations inherent in the species of different genera might be somewhat minimized by selecting seeds

from one family, or better, one genus. The seeds used in this investigation are of the legume family except the pumpkin which was included for further study as it was found in an earlier study to respond favorably to ammonium nutrition (**2**). The peanut was employed with the possibility that it might compare favorably with the pumpkin on the basis of the similarity in the chemical composition of the seeds (table I), thereby having the desired range of food reserves within one family.

The following seeds were chosen:

Pumpkin: *Cucurbita pepo* (Small Sugar variety), representing the cereal type; high oil–low protein reserves.

Peanut: *Arachis hypogea* (Spanish variety), possible representative of the cereal type in the legume family; high oil–low protein reserves.

White lupine: *Lupinus albus,* representing a possible intermediate between the above types and the yellow lupine; high hemicellulose—intermediate in protein reserves.

Yellow lupine: *Lupinus luteus:* PRIANISCHNIKOW's third type; low hemicellulose–high protein reserves.

The compositions of the kernels (seed coats removed from seeds) as determined during this investigation are presented in table I. Ether extract was

TABLE I

ANALYSIS OF SEEDS

DETERMINATIONS	PUMPKIN	PEANUT	WHITE LUPINE	YELLOW LUPINE
Weight of 100 seeds (gm.)	16.65	52.08	52.47	11.52
Weight of 100 kernels (gm.)	13.16	50.90	43.80	8.72
Moisture in kernels (per cent.)	4.20	4.90	6.18	6.14
Dry weight of 100 kernels (gm.)	12.61	48.41	41.10	8.18
	Percentage composition of kernels in terms of dry weight			
	%	%	%	%
Insolube nitrogen	5.39	5.16	6.80	9.26
Soluble nitrogen	0.22	0.24	0.53	0.29
Ether extract	50.27	46.71	13.10	9.05
Starch	——	6.69	——	——
Insoluble acid hydrolyzable polysaccharides	2.88	2.84	17.04	7.76
Reducing sugars	0.13	0.08	0.08	0.12
Sucrose	3.64	2.32	3.76	1.50

determined (**1**) on the kernels after grinding and drying at 80° C. The methods employed in the analyses are described in a following section.

Germination

The seeds were carefully selected for uniformity in size, and all those showing defects were discarded. At least 5 times as many seeds as were to be used in the experiments were germinated after preliminary treatment with 0.2 per cent. Uspulun for 20 minutes, and soaking for 2 hours in one-fifth strength nitrogen-free nutrient solution in the case of the pumpkin and white lupines. The peanuts were found to germinate better without Uspulun treatment. The yellow lupines were treated for 1 hour with concentrated sulphuric acid with subsequent washings in lime water. The germination was increased by this treatment from 15 to 90 per cent. with much more uniformity in rate of germination. After soaking for 2 hours, the seeds were placed in glass moist chambers on cellucotton kept nearly saturated with one-fifth strength nitrogen-free nutrient solution. The germination was improved by renewal of the air in the germinators at 12-hour intervals. By means of the above precautions, vigorous young seedlings free of bacteria and molds were obtained in three to four days, depending upon the species. Germination was carried out in the dark room (free of gas and laboratory fumes) in which the subsequent experiments were carried out (25°–26° C.). The young seedlings at the above mentioned stage were selected for uniformity (radicles 3 cm. in length) and transferred to the culture solutions. Analysis of the seedlings at this stage are presented in table II. The cotyledons were analyzed apart from the remainder of the seedlings. No attempt was made to analyze the plumules separately since these made up but a small fraction. Hence the term "roots" includes the plumules with the comparatively large and fleshy radicles. From 100 to 300 seedlings were analyzed in duplicate at this stage.

Nutrient solutions

Livingston (**15**) has stressed the need of much preliminary investigation to determine satisfactory salt combinations, concentrations, and pH before definite progress can be made in nutritional studies. In such studies complications arise; for an "optimum solution" varies among different species for a given stage of growth. Furthermore, environmental conditions alter the optimum solution for a given species and stage of growth. For example, an optimum solution for a normal seedling may be expected to differ materially from that for an etiolated seedling. It is evident that the choice of a suitable nutrient solution requires many considerations.

Just as Pirschle (**26, 27**) has recognized the importance of the interaction of various factors on plant response to pH, one thinks of the interionic and other interacting factors as certainly affecting the response of etiolated seedlings to ammonium salts. Hoagland (**8**), Lundegårdh (**16**), and Steward (**56**) have briefly reviewed some of the recent papers pertaining to the various relationships involved in mineral nutrition.

TABLE II

ANALYSIS OF GERMINATED SEEDLINGS AT THE BEGINNING OF EXPERIMENTS WHEN TRANSFERRED TO NUTRIENT SOLUTIONS
(WEIGHT PER HUNDRED SEEDLINGS)

Determinations	Pumpkin		Peanut		White lupine		Yellow lupine	
	Cotyledons	Roots	Cotyledons	Roots	Cotyledons	Roots	Cotyledons	Roots
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Fresh weight	23.2	18.0	82.5	28.2	110.0	22.2	22.1	10.0
Soluble solids	0.71	0.19	4.50	1.81	9.30	2.24	1.42	0.46
Insoluble solids	10.11	0.40	41.66	1.73	13.75	1.41	6.02	0.13
	Cotyledons	Roots	Cotyledons	Roots	Cotyledons	Roots	Cotyledons	Roots
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Insoluble nitrogen	635.4	22.3	1594.0	346.4	2341.0	90.1	629.2	24.2
Soluble N	42.9	13.6	337.5	246.0	326.0	80.0	101.5	33.8
Amino N	24.3	9.0	108.8	96.4	114.0	43.0	55.2	18.1
Amide N	7.2	3.4	32.0	24.0	32.0	35.0	27.0	10.4
Ammonium N	1.4	0.9	5.0	3.0	9.0	3.0	1.8	0.5
Reducing sugars	19	29	60	351	73	129	5	32
Sucrose	19	6	824	480	288	129	110	15
Starch	—	—	6333	961	—	—	—	—
Insoluble acid hydrolyzable polysaccharides	152	19	1150	22	5089	174	483	59

The question of pH of nutrient solutions requires serious consideration in connection with ammonium nutrition. The importance of the hydrogen-ion concentration as a determining factor in the absorption of ammonium and consequent response of plants has been stressed by PRIANISCHNIKOW, PIRSCHLE, MEVIUS and ENGEL, NAFTEL, and others (**17, 18, 21, 26, 27, 35, 36, 37**). In general, it has been shown that plants, under normal conditions, utilize ammonium most efficiently from a neutral or slightly alkaline medium; but the alkaline conditions are unsatisfactory because complications arise (precipitation, etc.). TIEDJENS (**58**) has indicated that pH values of 6.0–6.5 for ammonium nutrition with constantly flowing cultures were necessary for most satisfactory assimilation in the plant.

From preliminary trials the following salt combination on a partial volume-molecular basis proved suitable:

$(NH_4)_2SO_4$	0.006 M
$MgSO_4 \cdot 7H_2O$	0.004 M
KH_2PO_4	0.004 M
$CaCl_2$	0.004 M

A pH of 6.2 was obtained by the addition of N/10 NaOH. Proper aliquots of molar single salt stock solutions and the alkali were added to distilled water and diluted to volume just before using. The distilled water was allowed to come to dark room temperature before using. Traces of iron and boron were added. The control nutrient solution was made up in the same manner except that the ammonium sulphate was omitted.

CONTINUAL FLOWING CULTURES AND AERATION

As culture vessels 4-gallon glazed earthenware jars were used. Over each jar was placed a zinc galvanized wire net (¼-inch mesh) well coated with paraffin which served as an efficient support for the seedlings. A modification of the STAHL and SHIVE system of continuous renewal of nutrients (**55**) was adapted for use with these culture vessels. Five-gallon glass bottles calibrated at 15 liters were employed as supply and reception vessels. The rate of flow of nutrient solutions was so regulated as to allow the passage of 10 liters daily through each culture vessel, thus maintaining a constant pH. The drip solution was reused once after readjusting the pH to 6.2. The cultures were continually aerated with ammonia-free air (compressed air passed through a dilute sulphuric acid trap). The rate of aeration was such that the solution was kept in slow motion. This aeration treatment resulted in noticeably better root development. At the beginning of each experiment 250 young seedlings, selected for uniformity as previously described, were transferred to each of the 6 culture vessels (3 ammonium and 3 controls).

Methods of chemical analysis

Fractionation of plant organs

The seedlings at each sampling were separated into stems, cotyledons, and roots. In order to make more efficient use of the plant material, the lower one-third of the hypocotyl was included with the roots in all samplings. Fifty to one hundred seedings were sampled at each date of sampling. After obtaining fresh weights, without delay the plant material was minced into small sections (2–3 mm.) and transferred to Erlenmeyer flasks. Sufficient hot 95 per cent. alcohol was added to bring the final concentration of alcohol to 60 per cent. The contents were heated on the steam bath to gentle boiling for 20 minutes, and allowed to cool to room temperature before filtering.

Extraction

Considerable difficulty was experienced in obtaining complete extraction of the soluble nitrogen with 80 per cent. alcohol, especially in the case of the white lupines (containing large amounts of asparagine) from which, even after 10 extractions, the asparagine was not completely removed. This difficulty was overcome by reducing the alcohol concentration to 60 per cent. A lower concentration resulted in filtration difficulties, especially in cotyledon material of lupines. It has previously been pointed out by Denny (**6**) that unless the plant material is distinctly acidic there is little justification for employing $CaCO_3$ to prevent the possible hydrolysis of sucrose. In some preliminary studies it was found that young seedlings containing large amounts of sucrose, when extracted with and without the aid of $CaCO_3$, gave comparable results. Accordingly, it seemed best not to include $CaCO_3$; for its inclusion results in certain complications of analysis and may possibly render extracts sufficiently alkaline to result in a loss of ammonia (if present in appreciable quantities) during the removal of alcohol to obtain water extracts for nitrogen fractions. Eight to nine extractions at 6-hour intervals secured complete extraction in 2 to 3 days. After the first extraction the residue in the flasks were, in the subsequent extractions, covered with hot 60 per cent. alcohol, heated to gentle boiling for 5 minutes and allowed to cool before filtering. After the extractions were completed and the extracts diluted to a convenient volume, nitrogen fractions were determined without delay. Thus any changes which seem to occur in nitrogen fractions during storage of alcoholic extracts (**57, 63, 64**) were reduced to a minimum.

Analysis of extracts

Solids and soluble nitrogen were determined on suitable aliquots.

Soluble nitrogen fractions.—The alcohol was removed from suitable aliquots, the water suspension cleared, and made to volume (**2**).

Ammonium nitrogen was determined on 20-cc. aliquots of the water

TABLE III

CONCENTRATION OF AMMONIUM NITROGEN IN STEMS AND ROOTS OF AMMONIUM-NOURISHED SEEDLINGS ON FRESH WEIGHT BASIS

PERIOD OF GROWTH	PUMPKIN		PEANUT		WHITE LUPINE		YELLOW LUPINE	
	STEMS	ROOTS	STEMS	ROOTS	STEMS	ROOTS	STEMS	ROOTS
	%	%	%	%	%	%	%	%
3 days	——	——	——	——	——	——	0.007	0.009
6 "	0.012	0.022	0.017	0.023	0.010	0.009	0.027	0.020
9 "	0.025	0.027	——	——	——	——	——	——
12 "	——	——	0.019	0.025	0.021	0.036	——	——
14 "	0.044	0.045	——	——	——	——	0.045	0.045
16 "	——	——	——	——	0.046	0.087	——	——
18 "	——	——	0.027	0.030	——	——	——	——
30 "	——	——	0.037	0.032	——	——	——	——

extract in the Van Slyke-Cullen aeration apparatus. The aliquot was made 0.6 per cent. sodium hydroxide by the addition of 10 per cent. sodium hydroxide and aerated into 25 cc. of N/50 standard sulphuric acid, employing capryl alcohol to prevent foaming.

The ammonium-free aliquot was neutralized with glacial acetic acid added dropwise and an additional drop added to slightly acidify the mixture, which was then carefully transferred to a 50-cc. volumetric flask, the tube being carefully rinsed several times into the flask with small portions of distilled water. The flask was made to volume and the amino nitrogen determined on 2.5-cc. aliquots in the Van Slyke amino-nitrogen apparatus.

Ten-cc. aliquots of the cleared water extract were hydrolyzed (**25**), cooled, nearly neutralized with 1.5 cc. of 50 per cent. NaOH and allowed to recool.

TABLE IV

CONCENTRATION OF TOTAL SUGARS IN STEMS AND ROOTS OF AMMONIUM-NOURISHED SEEDLINGS ON FRESH WEIGHT BASIS

PERIOD OF GROWTH	PUMPKIN		PEANUT		WHITE LUPINE		YELLOW LUPINE	
	STEMS	ROOTS	STEMS	ROOTS	STEMS	ROOTS	STEMS	ROOTS
	%	%	%	%	%	%	%	%
3 days	——	——	——	——	——	——	0.214	0.050
6 "	0.276	0.114	1.383	0.639	0.625	0.510	0.006	0.009
9 "	0.062	0.042	——	——	——	——	None	0.003
12 "	——	——	0.877	0.269	0.136	0.040	——	——
14 "	0.006	0.012	——	——	0.009	0.017	——	——
16 "	——	——	——	——	——	——	——	——
18 "	——	——	0.424	0.220	——	——	——	——
30 "	——	——	0.040	0.010	——	——	——	——

The solution was then made neutral to neutral red, and without delay made 0.62 per cent. NaOH and aerated into 25 cc. of N/50 standard acid. This gave ammonium equivalent to ammonium plus amide nitrogen. In the light of the recent findings of PUCHER *et al.* (**28**), VICKERY (**61, 62**), STUART (**57**), SCHLENKER (**46**), DAVIDSON and SHIVE (**5**), HULME (**11**), RICHARDSON (**42**), TOTTINGHAM *et al.* (**59**), the present methods of analysis of N-fractions are seriously in need of further critical examination.

SOLUBLE CARBOHYDRATES.—Direct reducing sugars and sucrose were determined on cleared and deleaded aliquots of the alcoholic extracts (**2**). A modification of the TOMPSETT method (**23**) was employed in all carbohydrate determinations.

STARCH, INSOLUBLE ACID-HYDROLYZABLE POLYSACCHARIDES, AND NITROGEN.—Starch, insoluble acid-hydrolyzable polysaccharides, and nitrogen were determined on the residues (**2**). The peanut was the only species studied which contained starch. Dextrins were determined in the extracts of the peanut and included with the starch. Owing to the large sugar blank on the high grade commercial taka-diastase distributed by the Parke, Davis Co., it was found necessary to dialyze the preparation free of sugars before use (**7**). In the case of the peanut cotyledons, the residues were extracted with ether before determining starch.

EXPRESSING RESULTS.—It seemed that the results obtained in this type of investigation would in general be expressed most significantly on the basis of weight of the respective constituents per hundred seedlings. In order more conveniently to study the various trends on a comparable basis, the data were plotted graphically, thus facilitating a picturization of the relations in figures 1 to 8. The increases or decreases in the various constituents for the particular organs of a given species, resulting from ammonium nutrition, are readily shown in these figures. Certain conditions existing in the various species are more comparable when the constituents are expressed as percentage of fresh weight (tables III and IV). In order to make a comparable study of the seedlings with respect to growth response, ammonium absorption, and ammonium utilization in relation to the food reserves of the seed, certain constituents were expressed on a comparable basis of amount of constituent per 100 gm. of dry ungerminated kernels in figures 9 to 11.

SIGNIFICANCE OF RESULTS.—Having carefully selected the seeds and again the young seedlings for uniformity, variations in duplicate samples of the experimental plant material owing to individual variations of the plants were reduced to a minimum (often within the limits of error of the methods of chemical analysis (1 to 2 per cent.)), by the choice of a large number of individuals (50 to 100) per sample. Differences greater than 4 to 8 per cent. between the averages of four determinations per constituent of the two series respectively were considered significant.

Experimentation

Plan of experiments

Preliminary experiments were performed to determine the age of the seedlings of the various species at the time when starvation symptoms were apparent as the result of ammonium nutrition. Having thus determined the starvation periods for the various species, intervening sampling periods were chosen at suitable intervals in an attempt to obtain information useful in interpreting the conditions associated with ammonium injury.

Note that when the term "ammonium injury" is used in the following discussions it is not meant to imply that ammonium as such is injurious, as some writers seem to believe, but rather to convey in a convenient manner the complex of conditions associated with plants injured by ammonium nutrition. In short, the writer prefers to consider ammonium as only *one* of the various factors bringing about such pathological conditions. To be more specific, in the cases of the ammonium injury reported in the following discussions

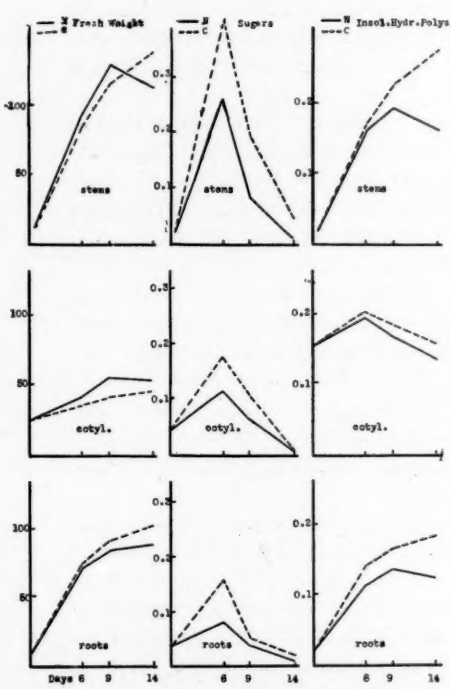

Fig. 1. Pumpkin. Fresh weights and carbohydrates in grams per hundred seedlings.

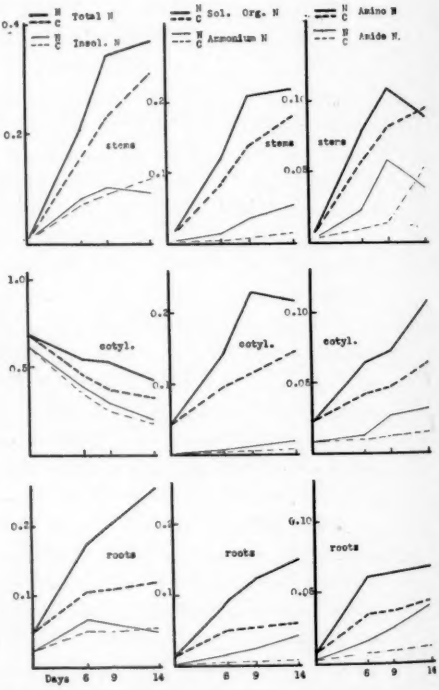

Fig. 2. Pumpkin. Nitrogen fractions in grams per hundred seedlings.

the writer prefers to consider that this condition is "injury due to extreme carbohydrate deficiency," since this would accurately express the truth; for, other conditions being favorable, including the presence of available carbohydrates, ammonium is *not toxic*.

EXPERIMENTS WITH PUMPKIN

The pumpkin seedlings were sampled at 6-, 9-, and 14-day periods. (The number of the days indicates the time elapsed from the date on which the young seedlings were transferred to the culture solutions.) The data obtained are graphically presented in figures 1 and 2.

A consideration of the fresh weight curves reveals the beneficial effects of ammonium on the growth of the stems and cotyledons during the earlier stages of growth. This favorable effect was previously noted (**2**). However, growth of roots was retarded. Similar growth relations were noted by REID (**41**) when etiolated seedlings were supplied with nitrates. The pumpkin differs materially from the other seedlings in that the cotyledons become very much expanded (growth) and succulent, and serve as very efficient photosynthetic organs in the light. At 14 days, the small leaves of the ammonium-fed epicotyls showed typical carbohydrate starvation symptoms (desiccation of the small young leaves).

The sugars were rapidly depleted in all organs by ammonium nutrition (fig. 1) with simultaneous decreases in amounts of hemicellulose. Sucrose, although present in appreciable amounts at the beginning of the experiments (table II), especially in the cotyledons as in the other species, disappeared at a much more rapid rate than the reducing sugars. Starch was absent from all tissues.

NITROGEN FRACTIONS.—The curves (fig. 2) show the remarkably rapid rate of ammonium absorption and utilization. In the early stage, protein formation especially in the roots was favored by ammonium nutrition, but not at the expense of the protein reserves of the cotyledons. It is of interest to note that the root development was retarded, even though protein formation was favored. These relations are to be expected since it has been repeatedly demonstrated by various investigators that root development is favored by higher carbohydrate conditions. In the intermediate stage (9-day) protein formation was retarded in the roots with a simultaneous increase in the stems, although not appreciable. Ammonium nutrition resulted in a sparing action on the reserve proteins at this stage. Amides strikingly had accumulated at this period while ammonium also accumulated in appreciable quantities in all organs. At the period of carbohydrate starvation (14 days) the synthetic processes were seriously disrupted as evidenced by the decrease in amino acids and amides. However, these conditions were just the reverse in the roots. The accumulation of amino nitrogen with a simultaneous

decrease in total soluble organic nitrogen in the ammonium-nourished cotyledons suggests that the amino acids accumulate at the expense of the more complex soluble compounds (polypeptides, etc.).

Experiments with peanut

The seedlings were sampled at 6, 12, 18, and finally at 30 days when ammonium injury was apparent (starvation symptoms—desiccation of the older leaflets). Control seedlings were grown for 40 days when starvation symptoms were evident similar to those observed in the ammonium-nourished seedlings. There was no nodule formation in either series of the peanuts and lupines, and no evidence of nitrogen fixation was obtained. As is shown in figure 3, the seedlings did not respond significantly to ammonium nutrition during the early stages. The differences in amount and concentrations of sugars between the two series were amplified as the seedlings approached starvation especially in the roots. Somewhat similar trends were also appar-

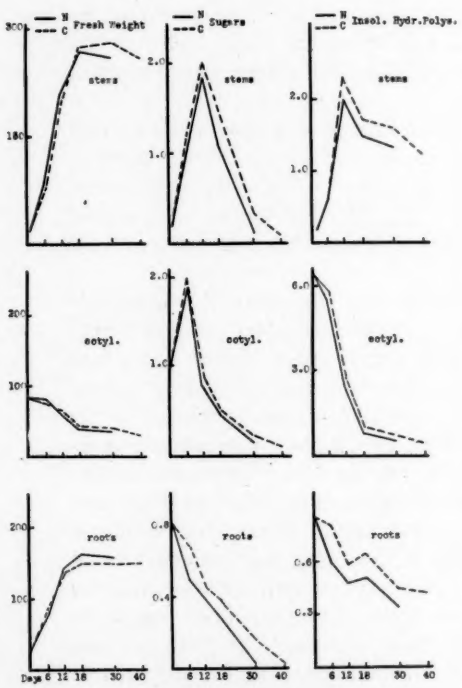

Fig. 3. Peanut. Fresh weights and carbohydrates in grams per hundred seedlings.

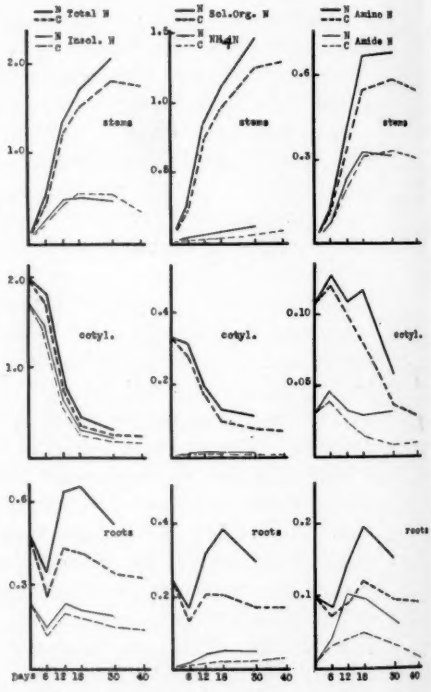

Fig. 4. Peanut. Nitrogen fractions in grams per hundred seedlings.

ent in the hemicelluloses and to a less extent in the starch in the cotyledons and roots. The trends in the nitrogen relations (fig. 4) were not as striking as those noted in the pumpkin. The initial drop in the nitrogen-fraction curves in the roots is due to the fact that the plumules and upper hypocotyls were not separated from the radicles at the initial sampling period. The relative rates of absorption and utilization of ammonium were considerably less than those noted in the pumpkin. Ammonium nutrition resulted in significant increases in protein at 12 days, but not at the expense of the protein reserves, owing to presence of adequate carbohydrate reserves. Marked increases in the soluble organic nitrogen fractions in the roots were favored by ammonium nutrition until the 18-day period. There seems to have been considerable translocation of the organic nitrogen from the roots to the tops in both series during the 18- to 30-day period. The decided increase in soluble organic nitrogen of the stems during this period seems to have been in part due to the movement of organic nitrogen from the cotyledons. The leveling of the organic nitrogen curves during this period suggests that the marked increase in the stems must have been caused by inward movement of organic nitrogen from the roots. Although the synthetic processes were materially inhibited during the carbohydrate starvation period (18 to 30 days) there seems to have been no serious breakdown of total organic nitrogen or of amides by ammonium nutrition even at the point of serious carbohydrate deficiency (fig. 4).

Experiments with white lupine

Seedlings were sampled at 6, 12, and finally at 16 days, when ammonium injury appeared characterized by typical starvation symptoms as previously described for the pumpkin (desiccation of the leaflets). The analytical data are presented graphically in figures 5 and 6. Ammonium nutrition resulted in better growth of roots, and inferior growth of tops, which was true throughout all stages. These relations were also noted in the yellow lupine experiments. Considerable enlargement of the lower hypocotyl resulted from ammonium nutrition. This enlargement was not caused by increased secondary thickening, but by enlargement of pith and cortical cells. Better development of lateral roots was favored by ammonium nutrition. Although significant decreases in sugars were noted in all organs of ammonium-nourished seedlings the hemicelluloses were not significantly less than in the cotyledons of the control except at starvation. The synthesis of organic nitrogen from ammonium proceeded rather smoothly during the early stages in all organs, especially the roots. However, ammonium nutrition resulted in a serious disruption of synthetic processes in the carbohydrate starvation period (12 to 16 days); amides were remarkably decreased in all organs of the plants, and ammonium accumulated in considerable quantities

in the stems and roots; however, the amino nitrogen simultaneously increased in these organs. During this period amides and amino nitrogen increased in the stems and cotyledons but decreased slightly in the roots of the controls.

Experiments with yellow lupine

Samplings were made at 3, 6, and finally at 9 days when carbohydrate starvation symptoms were apparent in the ammonium-nourished seedlings. Data obtained are presented graphically in figures 7 and 8. Very narrow differences in fresh weight of the various organs were obtained in the two series during the early stages (3 days). At 6 days the growth of the ammonium epicotyls was noticeably stunted, which is reflected in fresh weight difference in the stems in figure 7. This difference was much more pronounced at carbohydrate starvation (9 days). The depletion of sugars by ammonium nutrition was very marked at 3 days, especially in the roots, in which regeneration of hemicelluloses was materially hindered especially

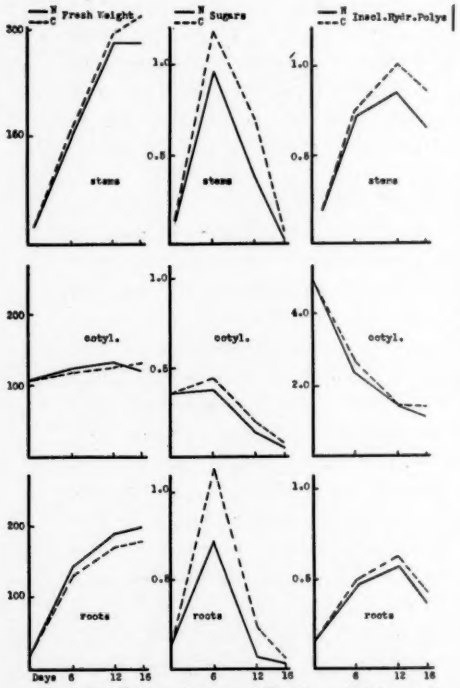

Fig. 5. White lupine. Fresh weights and carbohydrates in grams per hundred seedlings.

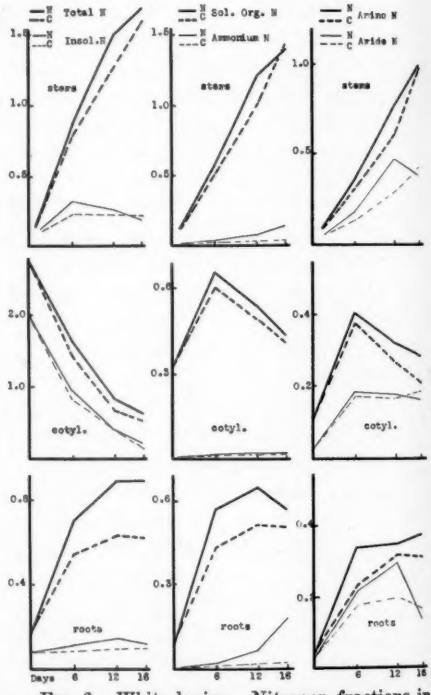

Fig. 6. White lupine. Nitrogen fractions in grams per hundred seedlings.

during later stages. Hemicelluloses seemed to have decreased in the stems during the 3- to 6-day period.

In the early stage, ammonium effected a significant increase in organic nitrogen, especially in the roots, where there was a significant increase in protein nitrogen. By the sixth day this increase in protein nitrogen had disappeared; however, during this same period there was a marked increase in amide and amino nitrogen which were favored by ammonium nutrition. This trend was not as great in the stems during this period. During the period of carbohydrate deficiency (6 to 9 days) the synthetic processes were seriously disrupted, especially in the stems, as a result for the most part of carbohydrate deficiency. Both amide and amino nitrogen decreased, and ammonium accumulated considerably. During this same period there was a remarkable increase in amide and amino nitrogen in the control stems.

These findings concerning the yellow lupine constitute a more complete story of the metabolism of the etiolated yellow lupine seedlings than the reports by PRIANISCHNIKOW and his co-workers (**29, 30, 31**). These investigators sampled the etiolated yellow lupine seedlings at ten days and did not make samplings at intervals during the life period. As indicated in figures 7 and 8, the life period of the etiolated yellow lupine seedlings supplied with ammonium was 9 days. After having determined the life period of the seedlings in preliminary trials, samplings were made at convenient intervals which were considered of interpretative value in respect to the various internal changes which occur during the progress of growth.

As indicated in the remarks concerning the other species of seedlings employed in these studies, a similar procedure of sampling the material for analysis was carried out. From the foregoing studies of ammonium nutrition, the following trends were noted in the various species of seedlings employed:

(1) In the early stages of growth, when available carbohydrates are abundant, ammonium is readily absorbed and utilized, resulting in protein synthesis which is especially evident in the roots. There is a tendency for growth of the seedlings to be favored by ammonium. Duration of these early stages varies considerably with the various species and seems to be associated somewhat with the type and amount of food reserves stored in the kernel.

(2) In the intermediate stages when available carbohydrates are less plentiful, although ammonium is absorbed and utilized, proteins are broken down and amides accumulate. During this period ammonium has little effect upon growth.

(3) During the carbohydrate starvation period, when available carbohydrates have become seriously depleted, ammonium is neither utilized, nor absorbed; proteins continue to be broken down; amides are decomposed; ammonium accumulates; and there may, or may not be, an increase in am-

monium nitrogen. There is a tendency for leaching of nitrogen from the seedlings to occur during this period as will be shown later. Carbohydrate starvation becomes progressively evident as characterized by cessation of growth followed by desiccation of the aerial organs. The accumulation of ammonium during this period is a clue to the breakdown of organic nitrogen compounds resulting from extreme deficiency of available carbohydrates.

Comparative study of seedlings

The comparative growth responses of the various species of seedlings supplied with ammonium are of interest with respect to their relationship to the food reserves. In figure 9 the fresh weights are presented on a comparable basis as a criterion of growth response.

During the early and intermediate stages of growth of the seedlings when sugars were plentiful (table IV), the ammonium absorption curve (fig. 10) closely paralleled the ammonium utilization curve (fig. 11) for the respective

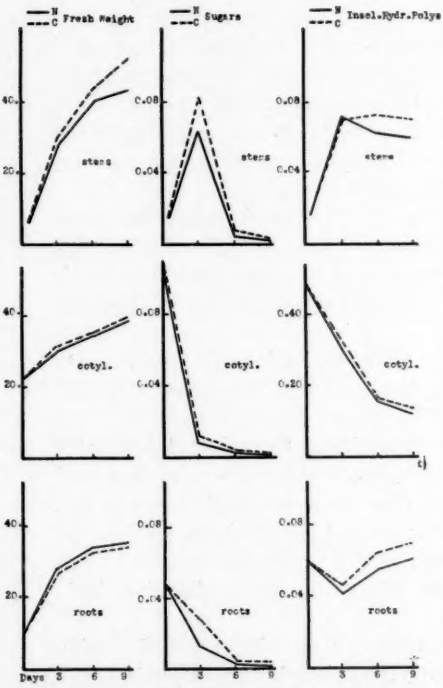

Fig. 7. Yellow lupine. Fresh weights and carbohydrates in grams per hundred seedlings.

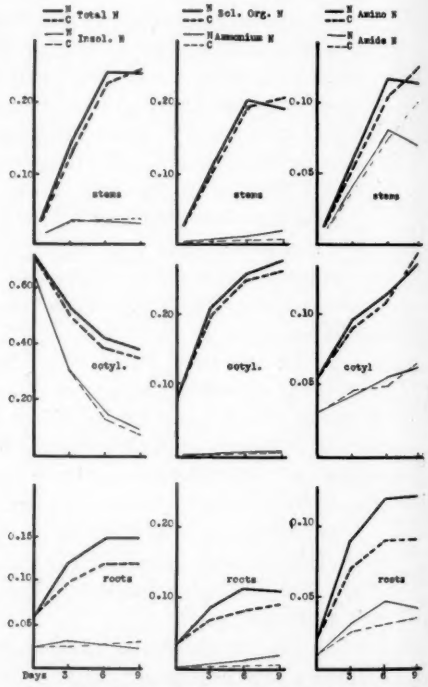

Fig. 8. Yellow lupine. Nitrogen fractions in grams per hundred seedlings.

species. During the later stages of growth as the available carbohydrate supply became exhausted (table IV), these curves diverged from each other due to the cessation of ammonium absorption in conjunction with the continued breakdown of the simple organic nitrogen compounds which results in ammonium accumulation (table III). The leaching of nitrogen from the seedlings during the carbohydrate starvation period is indicated in figure 10.

Although in the early stages of growth the concentrations of sugars in the stems and roots of the peanut seedlings are approximately six times the concentration of sugars in the stems and roots of the pumpkin seedlings (table IV), the rate of ammonium utilization by the peanut seedlings is about one-third the rate of ammonium utilization by the pumpkin seedling. This

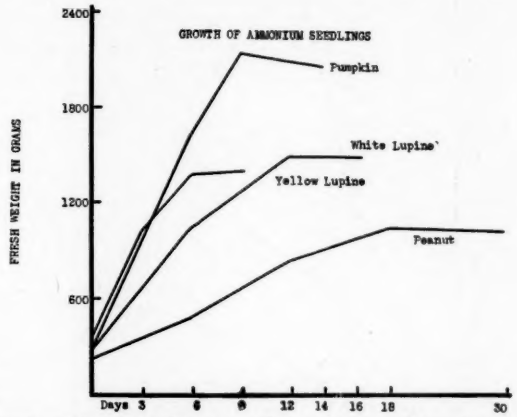

FIG. 9. Fresh weights of seedlings supplied with ammonium nitrogen (calculated on comparable basis as fresh weight in grams produced from a hundred grams of dry ungerminated kernels).

apparent inconsistency seems to be caused by inherent differences in the metabolic characteristics of the species not measured by the present chemical methods. It is difficult to understand why the peanut seedling should be so sluggish in its response to ammonium when the sugar concentration (table IV) in the actively growing seedling is so much greater than in the very responsive pumpkin seedling. The sluggishness of the peanut seedling cannot be attributed to an inferior absorption mechanism, for the concentrations of ammonium in the seedling of the peanut and pumpkin seedlings are approximately the same (table III). The behavior of the peanut seedlings, and their relatively long life period closely resemble the *Phaseolus* seedling (**2**) which represented the typical starchy legume type.

The apparent sluggishness of the peanut seedlings with respect to ammonium absorption and utilization seems to be associated in part with the

sluggish growth characteristics (fig. 8). On the other hand the remarkable response of the pumpkin seedlings with respect to ammonium absorption and utilization appears to be associated with the relatively rapid growth rate (fig. 8). This rapid growth rate seems to be related to the rapid conversion of the fatty reserves into sugars, which are utilized in growth and respiration but are not stored as starch, as occurs with a considerable portion of the sugars in the early stages of growth of the peanut seedlings.

The relative rates of respiration of the various species of seedlings should have thrown considerable light upon their comparative responses to am-

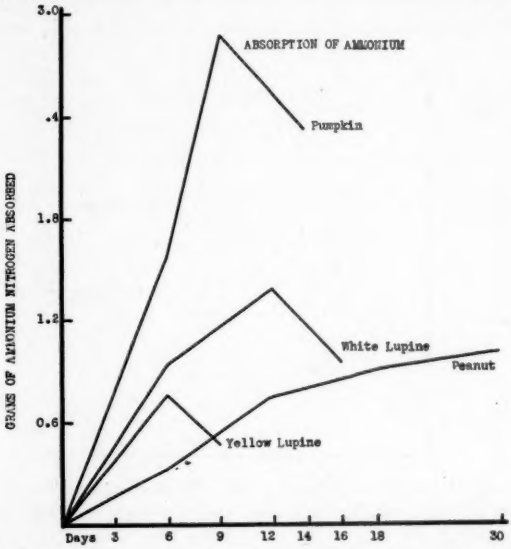

FIG. 10. Absorption of ammonium nitrogen by etiolated seedlings (calculated as difference in total nitrogen between the ammonium seedlings and control seedlings on comparable basis of a hundred grams of dry ungerminated kernels).

monium. Of special interest would be a comparative study of the respiration of the peanut and pumpkin seedlings of which the ungerminated kernels are high in oil. The rapid conversion of the oil to starch makes the peanut a starchy type, whereas there is distinctly no such conversion in the pumpkin seedlings which are so responsive to ammonium. A study of the respiration of the yellow lupine seedlings would be of interest in connection with the high protein reserve in the ungerminated kernels.

A comparative relationship of the soluble and total solids in the seedlings is presented in table V. In order more conveniently to make comparisons, the data pertaining to the solids in the seedlings have been purposely con-

densed to the form in which they appear in this table. Data pertaining to all of the species of seedlings appear for the 6-day period. And finally, data on solids at the period of extreme carbohydrate starvation, of the ammonium-nourished seedlings appear for the respective species. At the 6-day period the pumpkin seedlings increased in total solids, but markedly decreased thereafter. There seems to be no consistent trend with respect to the effect of ammonium upon the total solids. In the case of the pumpkin, however, ammonium resulted in a very significant decrease in total solids at the 14-day period. This seems to be the resultant of increased respiration because of the favorable effect of the ammonium upon the growth of these seedlings. Of

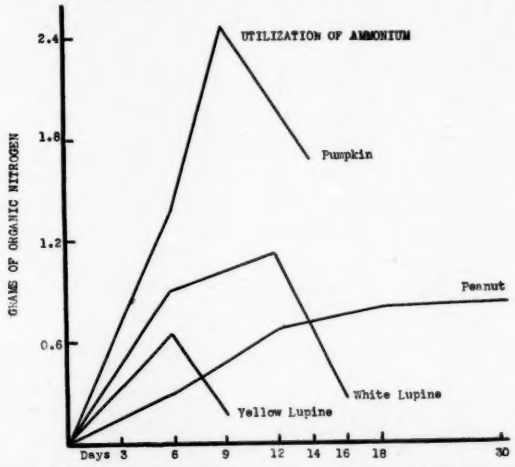

FIG. 11. Utilization of ammonium nitrogen by etiolated seedlings (calculated as difference in organic nitrogen between the ammonium seedlings and control seedlings on comparable basis of a hundred grams of dry ungerminated kernels).

the respective species, the loss of total solids during the life period was greatest in the case of the peanut, which seems to be associated with its long life period and the nature of the food reserves in its kernels.

General discussion

A few remarks in regard to the present status of the nitrogen relationships in seedlings are now presented for the purpose of orientation. One of the most striking observations has been the accumulation of amides in shoots of growing seedlings (especially of the Leguminosae), when germinated in darkness for two or three weeks. This observation has been the subject of much discussion in recent years. In some seedlings, the proportion of amides to amino acids is fairly uniform at certain stages of germination, while at other

TABLE V

SOLIDS IN SEEDLINGS AS PERCENTAGE OF DRY UNGERMINATED KERNELS (N = AMMONIUM-NOURISHED SEEDLINGS; C = CONTROL SEEDLINGS)

PERIOD OF GROWTH	PUMPKIN		PEANUT		WHITE LUPINE		YELLOW LUPINE	
	SOLUBLE SOLIDS	TOTAL SOLIDS	SOLUBLE SOLIDS	TOTAL SOLIDS	SOLUBLE SOLIDS	TOTAL SOLIDS	SOLUBLE SOLIDS	TOTAL SOLIDS
	%	%	%	%	%	%	%	%
6 days N	46.7	104.6	22.6	96.4	45.9	95.9	47.8	80.8
C	44.7	101.6	22.8	96.6	45.4	93.0	49.4	82.8
9 days N	—	—	—	—	—	—	49.5	79.5
C	—	—	—	—	—	—	49.4	79.5
14 days N	36.2	81.3	—	—	—	—	—	—
C	32.9	87.8	—	—	—	—	—	—
16 days N	—	—	—	—	40.1	73.5	—	—
C	—	—	—	—	36.9	72.7	—	—
18 days N	—	—	33.6	77.8	—	—	—	—
C	—	—	32.2	77.9	—	—	—	—
30 days N	—	—	24.6	67.8	—	—	—	—
C	—	—	24.4	68.8	—	—	—	—

stages amides prevail. Recently PUCHER (**28**) and VICKERY (**62**) have demonstrated the widespread occurrence of glutamine in seedlings. Seedlings of legumes are rich in asparagine, while high oil-containing seeds, such as the sunflower, pumpkin, and castor bean, produce considerable amounts of glutamine. It has been shown that the amides accumulate in greater amounts than can be accounted for by direct hydrolysis of reserve proteins. Recently KLEIN and TAUBÖCK (**12, 13**) have emphasized the importance of basic amino acids which accumulate in considerable amounts in certain types of seeds on germination, with special attention given to arginine. Little is known concerning the interrelationships and significance of these facts.

SCHULZE (**47–53**) and PRIANISCHNIKOW (**29–34, 39**) have independently conducted a long series of investigations in which attempts were made to throw light on these phenomena. Both agree that much asparagine is formed during germination, and that under favorable conditions it is used in protein synthesis. Ammonium is formed by deaminization, probably by oxidation of amino acids. The origin of succinic acid, which they postulate plays an important rôle in asparagine formation, is somewhat obscure. SCHULZE (**50**) is inclined to believe that amino acids → ammonia breakdown with subsequent formation of asparagine is to a large extent an essential process, and that asparagine is the chief source of nitrogen for protein synthesis.

PRIANISCHNIKOW (**29–37, 39**) regards asparagine chiefly as a by-product in which form the surplus, and harmful though valuable ammonium may be stored for future use. He believes that the accumulation of amides is dependent upon a proper carbohydrate-nitrogen balance. When excess carbohydrates are present, as in photosynthesis, asparagine disappears owing to favorable conditions for protein synthesis. When this condition exists, asparagine serves as a translocatory form of ammonium and is utilized in protein synthesis. CHIBNALL is in favor of this view (**3, 4**). When a reasonable amount of carbohydrates is present, as in the early growth stages of etiolated seedlings, there is an accumulation of amides, for conditions are not favorable for maximum protein synthesis. But when extreme deficiency of carbohydrates is obtained, there is no opportunity for the accumulation of asparagine, owing to lack of oxidation products upon which asparagine synthesis depends. Under these latter conditions, ammonium accumulates, thereby becoming toxic. The writer seriously questions the validity of such an inference regarding the toxicity of ammonium, because it may accumulate considerably in plants without injury (**14, 44**). He prefers to think of the pathological condition during which ammonium accumulates as caused by an unfavorable complex of conditions in which depletion of available carbohydrates plays a predominant rôle, and that the accumulation of ammonium is probably a resulting phenomenon, but not the cause of the condition.

MURNEEK (**20**) in recently reviewing the rôle of asparagine and related substances in plants has not seemed to have critically evaluated the findings and conclusions of PRIANISCHNIKOW. It seems that, although amides accumulate under certain conditions, there is little justification for assuming that amides serve as efficient detoxicants of ammonium. It is believed that further studies of the so-called "acid plants" (**14, 43, 44**) subjected to ammonium nutrition should stimulate new lines of thought in regard to these phases of nitrogen metabolism. The writer prefers to consider the accumulation of asparagine to be a resultant of certain carbohydrate-nitrogen relations and that it really does not serve a purpose in the detoxification of ammonium.

Owing to the lack of a definite understanding of nitrogen relations existing in normal seedlings, it becomes very difficult to make a satisfactory analysis of the results obtained in this investigation. Accordingly, some interpretations and generalizations in regard to the conditions brought about by ammonium nutrition in the etiolated seedlings are of necessity partially speculative. In order to grow plants under controlled conditions, they must necessarily be placed under somewhat artificial conditions. Even though the seedlings employed in this study were grown under extremely artificial conditions (**40**), it is believed that a proper analysis of the controlled environmental factors in conjunction with a careful consideration of the differences brought about by the variable (ammonium supply), should throw light upon

certain phases of plant metabolism which may not otherwise be obtained. From this viewpoint, this study was deemed justifiable in the light of the present confusion regarding the rôle of certain nitrogen compounds in the normal metabolism of plants.

In these experiments it was found that growth response of the various seedlings supplied with ammonium salts was in part dependent upon the nature of the food reserves. In this connection, REID (**41**) has mentioned some of the practical implications arising from studies of the response of etiolated seedlings to nitrogen supplied in the form of nitrates. Owing to the wide differences in the morphological characteristics, as well as to the complex hereditary tendencies inherent in the various species, it becomes somewhat far-fetched or questionable definitely to assign the different types of response (growth and certain internal conditions measurable by present chemical methods) to the type and amount of food reserves existing in the kernel. Certainly the hereditary tendencies in conjunction with modified physico-chemical relationships must play an important part in determining the responses of various seedlings to ammonium nutrition. The trends noted in these species would in no wise be expected to occur in the "acid plants" of RUHLAND and WETZEL (**43, 44**), especially with respect to the amide relations.

A further complication in making certain generalizations in such an investigation is the lack of knowledge as to the exact nature of the food reserves in the seeds; for example, ether extract and hemicelluloses are empirical terms assigned to groups of substances (more or less heterogeneous) about which little is specifically known. MILLER (**19**) gives a comprehensive historical review of the earlier investigations pertaining to the ether extract as storage material in the seeds and the changes it undergoes during germination. In this connection he (**19**) has studied quite extensively the changes occurring during the germination of sunflower seedlings. Little work has been done on hemicelluloses of seeds and seedlings, aside from the studies carried out by SCHULZE (1889–1910). Reserve protein is an ambiguous term, in that it is so closely interrelated to non-reserve protein that a differentiation of the two forms as they occur in the storage organs is beyond the scope of present methods of chemical analysis (**22**). Furthermore, little is known concerning the nature and rôle of non-protein nitrogen in the seed and seedlings, especially the "rest nitrogen."

The question as to the mechanism of ammonium absorption by plants has received but little consideration. NEVIUS (**17, 18**) believes that ammonium absorption increases in neutral or slightly alkaline solutions, owing to the greater degree of hydrolytic cleavage of the salts and correspondingly greater ammonia tension, which determines to a large extent the physiological effects of ammonium salts. Furthermore, he states that the injury may result from

a too rapid accumulation of ammonium under neutral or slightly alkaline conditions. NAFTEL (**21**) believes that ammonium alters the colloidal complex of the protoplasm which in turn affects the rate of absorption of ammonium. The study of interionic absorption, to say the least, involves many complications (PIRSCHLE, **26, 27** and NEVIUS, **17, 18**). Although in accordance with absorption principles, these explanations are quite theoretical and the assumptions call for experimental verification. HOAGLAND (**8**), LUNDEGÅRDH (**16**), and STEWARD (**56**) have briefly reviewed some recent papers pertaining to ionic absorption. HOAGLAND and BROYER (**9**) believe that the absorption of ions is dependent upon metabolic activity. This view seems highly speculative. HOAGLAND (**8**) also emphasizes the importance of the buffering system of plant sap as a factor in ionic absorption. PRIANISCHNIKOW (**36, 37**) has recently shown that the rates of absorption of ammonium and nitrate by etiolated seedlings from single salt solutions of ammonium nitrate varies considerably with the pH of the culture medium and the age of the seedlings. However, no attempt was made to determine the extent of utilization. The author questions the value of such experiments in which single salt solutions are employed.

One thinks of the ammonium ion, after having entered the root of seedlings, as affecting directly or indirectly innumerable processes which in turn have a direct bearing on metabolism and growth. Various physico-chemical relationships in the cellular structure must be altered by the ammonium ion, such as permeability, translocation, pH, and interionic relations within the plant tissue. HOLLEY *et al.* (**10**) have shown that ammonium definitely reduced calcium absorption, which in turn may affect materially the mobilization of carbohydrates and other substances. The physico-chemical effects of ammonium may be expected to modify many metabolic processes such as hydrolytic reactions (hydrolysis of reserves, etc.), translocation of hydrolytic products, mobility and lability of sugars, amino acids, etc., respiration (in this investigation certain relations concerning dry weights seemed to suggest that respiration was little affected by ammonium nutrition under the condition of these experiments), and synthetic processes, which in turn largely determine the organo-chemical response of seedlings to ammonium nutrition.

In this nitrogen investigation of etiolated seedlings supplied with ammonium, injury resulted from the extreme deficiency of available carbohydrates. The concentration of ammonium did not increase greatly when carbohydrate deficiency became very severe; however, during this period leaching of nitrogen occurred from all species studied except perhaps the peanut seedlings. KULTZSCHER (**14**) concludes that the reaction of the cell sap is important in the storage of ammonium. More information is seriously needed concerning the extent and conditions under which ammonium may accumulate in plants without injury in order to more satisfactorily evaluate

the rôle of ammonium in plants. More extensive organo-chemical investigation supplemented with histological and physico-chemical studies are needed in order to obtain a more satisfactory conception of the conditions associated with so-called "ammonium injury" which in a large measure seems to be caused by the depletion of available carbohydrates.

Summary and Conclusions

1. A brief resumé of PRIANISCHNIKOW's investigations pertaining to the rôle of ammonium in the nitrogen metabolism of etiolated seedlings and a critical analysis of his methods are included in the introduction.

2. With the adaptation of improved methods this investigation involves a further study of the effects of ammonium nutrition on the carbohydrate and nitrogen relations of etiolated seedlings. Seedlings of the following species were employed:

Cucurbita pepo (high oil reserve) pumpkin
Arachis hypogaea (high oil reserve) peanut
Lupinus albus (high hemicellulose and protein reserve) white lupine
Lupinus luteus (high protein reserve) yellow lupine

The seedlings were fractionated into stems, cotyledons, and roots for the analyses which were made at suitable intervals until the plants showed carbohydrate starvation symptoms.

3. In this investigation the conditions which resulted during ammonium nutrition cannot be ascribed to physiological acidity. In the light of modern principles of plant nutrition the methods employed by PRIANISCHNIKOW render the basis for his classification of seedlings unjustifiable.

4. The rate of absorption and utilization of ammonium was most rapid in the case of pumpkin. Although the oily reserve was rapidly converted into sugars no starch was formed therefrom. There was very little reversion of the sugars to fat in the seedlings. A marked depletion of sugars was associated with the rapid utilization of ammonium. Growth was favored by this association in the earlier stages during which time protein formation or regeneration was favored by ammonium nutrition. At the stage of extreme carbohydrate starvation (fourteen days) the protein and amide fractions were materially diminished in the stems while ammonium accumulated.

5. Although the food reserves of the peanut kernel are somewhat similar to those of the pumpkin, the response of the peanut seedlings to ammonium was very sluggish. During the early stages of germination and growth of the seedlings the oily reserve was rapidly converted into sugars, and in turn much of the sugar was converted into starch in the cotyledons and in the primary roots. At the time of carbohydrate starvation starch had entirely disappeared from the primary roots; however, a small amount of starch remained in the cotyledons. Even though the peanut kernels were high in oil

reserve the peanut seedlings behaved as physiological starchy legumes. The accumulation of amides favored by ammonium nutrition in the roots during the early stages was associated with a decrease in the sugars. Amides later decreased as sugars were further depleted. The ammonium peanut seedlings showed carbohydrate starvation symptoms at thirty days, while the controls showed similar symptoms at forty days.

6. Ammonium was utilized especially by the roots until the twelfth day in the case of the white lupine. Ammonium nutrition resulted in a marked depletion of amides in all organs during the carbohydrate starvation period (12 to 16 days) due to the exhaustion of the sugar supply resulting from earlier utilization of ammonium. Ammonium rapidly increased during the carbohydrate starvation period.

7. The yellow lupine seedlings utilized ammonium in the early stages (3 and 6 days). During the carbohydrate starvation period (6 to 9 days) ammonium was neither utilized nor absorbed. The amides were broken down and ammonium accumulated as a result of extreme depletion of available carbohydrates.

8. All the seedlings studied absorbed and utilized ammonium during early stages of germination while the synthetic processes were disrupted during the carbohydrate starvation periods when ammonium accumulated as a result of the breakdown of organic nitrogen because of the exhaustion of available carbohydrates. Ammonium injury appeared to be associated with carbohydrate depletion but is not ascribed to any specific condition.

9. In this investigation it appears to have been demonstrated that there are at least three distinct internal conditions with intergradations which are associated with the response of the etiolated seedlings to ammonium nutrition, namely:

(1) In the early stages of growth when available carbohydrates are abundant ammonium is readily absorbed and utilized resulting in protein synthesis which is especially evident in the roots. There is a tendency for growth of the seedlings to be favored by ammonium. The degree of response and duration of these early stages varies considerably with the various species and seems to be associated somewhat with the type and amount of food reserves stored in the kernel.

(2) In the intermediate stages when available carbohydrates are less plentiful, though ammonium is absorbed and utilized, proteins are broken down and amides accumulate. During this period ammonium has little effect upon growth.

(3) During the carbohydrate starvation period, when available carbohydrates have become seriously depleted, ammonium is neither utilized, nor absorbed; proteins continue to be broken down; amides are decomposed; ammonium accumulates, and there may, or may not be an increase in amino

nitrogen. There is a tendency for leaching of nitrogen from the seedlings to occur during this period. Carbohydrate starvation becomes progressively evident as characterized by cessation of growth followed by desiccation of the aerial organs. The accumulation of ammonium during this period is due to the breakdown of organic nitrogen compounds resulting from extreme deficiency of available carbohydrates.

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STRUCTURE OF THE STARCH GRANULE

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Introduction

The purpose of this paper is to present an hypothesis regarding the anatomical[1] structure of natural starch granules. The hypothesis itself is not new; it is a combination of parts of older hypotheses, but with the introduction of some concepts new for starch, though not new in themselves. The hypothesis, reduced to its simplest terms, will first be stated without proofs at the risk of biasing the reader against it. The physical properties of granules, so far as pertinent, will be examined to determine whether the hypothesis is useful for understanding them.

The hypothesis is based on certain observations recorded in the literature or newly presented here. These observations are:

1. The starch granule may be made to swell and shrink by varying its water content.
2. At ordinary temperatures, swelling in water is limited; at higher temperatures, it is great and may lead to ultimate dispersion of most of the granule substance (gelatinization).
3. The granule, if mechanically injured, *i.e.*, if it is chipped or cracked, swells greatly in cool water at the site of injury, and only there.
4. The granule is insoluble in cold water.
5. The granule, if mechanically injured, is more or less soluble in cool water by partial dispersion of the swollen portion.
6. The natural granule consists—at least in large measure—of material in orderly arrangement and exhibits a characteristic x-ray diagram. The diagram may be changed by causing the granule to swell, and under some circumstances the process upon which the change in diagram depends is reversible.
7. Completely anhydrous starch exhibits no x-ray diagram.
8. Thoroughly ground-up and mechanically disintegrated starch exhibits no x-ray diagram.

THE HYPOTHESIS

The peculiar anatomical organization of the intact granule restrains its swelling beyond a certain point in cool water. This restraining anatomical structure is the parallel arrangement of the long-chain macromolecules of which the starch granules' substance in large part consists. They are tied together in crystallites which are oriented radially in the granule. More-

[1] This word is used here to indicate that this paper does not deal with the finer molecular structure nor with the arrangement of the atoms in the molecule.

over, the starch-chain molecules possess an inherent tendency, probably due to intramolecular attraction of side groups for one another, to crumple or fold so as to reduce their own length. In crumpling, the macromolecule increases its diameter laterally; it thickens. In the natural granule containing adsorbed moisture, the macromolecules are not crumpled or shortened to their utmost because, being surrounded on all sides by other molecules which are also trying to thicken, room for complete contraction is lacking. The molecules at the same time endeavor to adsorb water, whereby they are also thickened. Thus, in swelling, a lateral pressure is exerted on the chains or bundles of chains, the micelles, which not merely prevents them from contracting but may even lengthen them so that the granule enlarges its diameter. In a sense, therefore, the macromolecules of a granule containing adsorbed moisture may be said to be stretched. They differ from the macromolecules in a mechanically stretched wool fiber in that the stretching force is a lateral pressure applied from all sides instead of a pull in the direction of the long axis of the fiber. The starch macromolecule is lengthened by squeezing, as one flattens a sheet of corrugated-iron roofing by lateral pressure; wool macromolecules are lengthened by pulling, as one straightens a coil of soft wire. The micelles are prevented from swelling to capacity, because there is not room for them to thicken as much as would be necessary if they sorbed or associated water to the maximum possible for them in a mechanically free state. Therefore, the swelling of granules in cool water is limited. Conversely, as a granule is dried, the transverse diameters of the macromolecules become smaller; the molecule shrinks laterally. The macromolecules crowd against one another with less force; space between them or between the micelles is made available to the extent that the water leaves. The granule shrinks until, in the completely anhydrous state, the chains approach a state of complete contraction.

So much for the hypothesis. We have now to present the evidence upon which it is based, and to test its usefulness in interpreting the physical properties of starch.

Supporting evidence

Swelling

It has been established conclusively by actual measurement that the starch granule shrinks when dried, and swells again when moistened (**3, 64,** p. 18; **77**). Meyer (**64**) found that drying at 20° C. for only 2 hours shortened the longest diameter of a potato-starch granule, freshly prepared and washed in water, by 15 per cent., and this observation has been confirmed in the writer's laboratory. Further drying at 90° C. for half an hour reduced its diameter by only 4 per cent. more. Additional final drying at 110° C. failed to produce further shortening. Total shrinkage is clearly very con-

siderable and therefore difficult to explain, as we shall see, if starch is composed of chain macromolecules that behave like those of fibers.

As one would expect from the capacity of the granule to swell in cool water, granules saturated with moisture are quite elastic. They can be deformed by mechanical pressure; but their greatest possible linear increase is only 3 to 6 per cent. Though very elastic, they do not stretch much (**70**, p. 39).

Like textile fibers (**8**), starch seems to exhibit hysteresis with respect to moisture sorption. Only moderate shrinking is promptly reversible. Sharply dried granules, if exposed to water vapor, do not swell rapidly back again to their original volume (**3, 107**).

Moreover, even moderate drying diminishes the power of starch granules to swell when boiled. They do not—at least for some time—swell to the same size that they would have reached had they not been dried (**3**).

Drying raises the gelatinization temperature (**56, 73, 76**) and affects other properties as well.[2] The length of time starch has been wet also seems to be of influence upon the gelatinization temperature (**81**). If drying is carried to the extreme, but only then, the x-ray diagram of the granule disappears;

[2] The effect of treatment with alcohol and ether is probably also to be attributed to dehydration. Wheat starch treated only with ether yields pastes of much greater mobility than the same starch sample treated with both alcohol and ether (**55**).

Not merely does thorough drying alter the swelling and water-sorption capacity, but starch thoroughly dried is said to be more easily hydrolyzed than it is before drying (**79**). Possibly this is because drying tends to produce rifts and cracks. Injuries tend to cause some of the starch substance to disperse (see pp. 298, 318). This interpretation finds support in the observation of NÄGELI (**70**, pp. 52, 63, 107) that the rifts and cavities grow larger in dry potato starch when it is placed in water. MEYER (**64**, pp. 96–99) and BARANETZKY (**13**) have observed that dry grains are more quickly affected by diastase than freshly prepared ones, because fissures in them facilitate the entrance of the enzyme. MEYER also observed that potato starch, which is slowly attacked by diastase, is attacked more readily if the outer layer is broken.

BROWN and HERON (**17**) and MAQUENNE (**59**) have shown that mechanically injured granules are attacked with great rapidity by diastase, observations which have been confirmed by GRIFFING (*cf.* **2, 76**).

These effects of drying are therefore probably due to rifting of some of the granules which renders them partially soluble (see p. 309, and *cf.* also **87, 88**).

Dehydration may also account for some of the differences between root and seed starches. In roots, the starches are never dry; in many seeds, for example the small grains, the starch may be quite dry, for the moisture content may sink as low as about 6 per cent. Indeed, the condition of the starch may perhaps account for some of the peculiar properties of flour made from such very dry wheat. Clearly, the starch of wheat harvested with a moisture content of 18 per cent. is not in a condition identical with that of wheat harvested with a low moisture content, say 8 per cent. It may be that some of the granules of very dry wheat, such as is harvested in semi-arid countries like California and British India, are rifted and thus influence the diastatic power of flour made from them.

incompletely dried starch retains its x-ray diagram (**47, 48, 65,** p. 1111; **66**), some starches losing their x-ray diagram more readily than others (**47, 48**).

It has long been known that the temperature at which the granules of a given starch species swell is distinctly different for granules of different size. The phenomenon has recently been studied in some detail by KATZ and HANSON (**49**). They concluded that each individual starch granule has a growth organization (*gewachsene Struktur*) which resists swelling and which must be overcome if gelatinization is to take place. No doubt there must be some such organization, but it may well be substantially the same in all granules of the same species and yet permit granules of different sizes to swell at different temperatures. Some years ago, the writer (**3**) pointed out that the relation of the mass of swelling material to the surface area of granules must in itself be a factor in determining the fact that in the same sample large granules tend to gelatinize more easily than small ones. The volume of a sphere or its mass increases as the diameter increases much faster than does its surface area. Therefore, given structure of identical rigidity in two granules of different diameter, the larger one should swell more readily than the smaller. It should swell sooner as the temperature is raised, because it will begin to exert enough intragranular pressure to distend the restraining structures of the granule before these have been softened to as great a degree as they must be to permit the smaller granules to swell at all. This explanation is supported by the fact apparently first pointed out by NÄGELI (**70,** p. 68) that in granules with very eccentric lamination swelling begins sooner on their eccentrically larger side, while the smaller side is still unchanged.[3] Indeed in very eccentric granules swollen in dilute potassium hydroxide the eccentrically larger side may swell around the still unswollen hilum end so that the latter forms a depression until it, too, swells and the whole granule becomes more or less uniformly inflated (**70,** p. 77). The pressure exerted in swelling, however, must be unequal in such granules on the two sides, for the reason given above. It must be greater on the larger side and swelling must be greater there than on the smaller side.

MECHANICAL INJURY

That mechanically injured granules swell in a different manner was first observed by SCHLEIDEN (**84**), and has often been confirmed. A cracked or chipped granule swells at the site of injury and only there. HARRISON (**36**) has reproduced a photomicrograph of such a granule. Injured granules,

[3] KATZ and HANSON (**49**), however, report that in every sample there are a few granules which partially resist gelatinization when heated to the threshold temperature at which gelatinization begins. Only a part of them becomes isotropic and stains with Congo red. This is commonest for eccentric granules. The eccentrically larger side may remain ungelatinized even when the smaller side has already undergone this change.

however, do not swell in anhydrous liquids such as pure glycerol; though in reagents like potassium hydroxide swelling begins at the injured spot (**11**). In water, swelling does not extend into the interior of the granule and then gradually throughout the whole of the granule as one would expect if a limiting membrane were the only mechanism that prevented the swelling of uninjured granules. Nor does the swelling spread circumferentially to involve the material between the laminae that have been fractured, as one would expect if the laminae were separated by membranes. These phenomena are particularly striking in granules broken in two by a fracture passing through the hilum. The two halves swell and disperse only over the surface of the fracture and for a short distance beneath. The halves do not swell *en masse*. They retain a normal appearance and their optical properties do not change except at the swollen fractured-surface region (**31**). HUSS (**41**), moreover, has demonstrated that triturated potato starch suspended in water occupies a greater volume than an equal weight of uninjured starch because a part of it is swollen. Indeed, the degree of swelling depends upon the extent of the injury, for the swollen portions do not wholly disperse. An extensively injured granule may swell to very nearly the size it would have reached if boiled in the uninjured state, as was established by measurement with a micrometer eyepiece (**31**). This is apparently because the natural granule contains α-amylose (amylopectin),[4] which swells in cold water but does not disperse.

[4] There is much confusion in the nomenclature. It seems generally agreed that all starches contain a substance easily dispersed in water. For it, MEYER (**64**) introduced the term, β-amylose, which is still very generally used, though sometimes this substance is simply termed amylose.

Many starches also contain insoluble material, which MEYER termed α-amylose, but later MAQUENNE termed it amylopectin. At that time, it was not yet known that some starches contain combined fatty acid of high molecular weight, while others contain none but usually instead phosphoric acid. The presence of phosphoric acid was discovered first, and it was believed that its presence was characteristic of α-amylose or amylopectin, so-called. SCHOCH (**85, 105**), however, has shown that in potato starch there is no insoluble fraction. According to this view, amylopectin is not a chemical individual, but merely β-amylose esterified to different degrees with phosphoric acid, or β-amylose in different degrees of association esterified with phosphoric acid. KADEN (**44**), working under the direction of C. J. LINTNER, has questioned its very existence. Moreover, some native starches apparently contain no insoluble material at all, but consist wholly of β-amylose (**38**).

The fatty-acid compound occurs preformed in cereal starches; it is insoluble. It is the only insoluble substance whose existence preformed in native starches has not been questioned. It follows that it is the only substance generally admitted to occur in native granules which conforms to the specifications as regards insolubility laid down for amylopectin by MAQUENNE, who introduced this term.

It seems, therefore, inadvisable to continue to employ the term amylopectin, for not all starches contain preformed insoluble material, though they may contain combined phosphoric acid. Further, amylopectin, so-called, has none of the chemical characteristics

The swelling of injured granules seems to be quite analogous in character to that of granules gelatinized by heat. The two sorts of swollen granules behave similarly with respect to staining and they exhibit similar optical properties. Tannin acts only upon that part of the granule which is injured in the same manner in which it acts upon boiled granules (**14**). Intact granules do not stain with such dyes as Congo red (**54**), which are among the colloidal dyes, though granules are stained by other dyes. FISCHER (**27**) found that, in granules impregnated with these dyes, picric acid precipitates the dyes in crystalline needles arranged radially. This may be taken as evidence of radial structure of the granule.

Injured granules, however, stain easily with the dyes, like Congo red, that do not stain intact granules, the staining being restricted to the injured part (**27, 31, 41, 112**). Heat-gelatinized granules stain like the swollen, injured portions of unheated granules, but the staining tinges the whole of the granule (**30**). HUSS (**41**) has, indeed, proposed the use of this phenomenon as an indicator to fix gelatinization temperatures. Congo red stains only the swollen parts of granules; allowing the granule to remain in the dye solution for 48 hours does not result in the penetration of the dye much, if at all, beyond the swollen area. The dyed material appears granular, taking the dye unevenly, whereas granules gelatinized by heating stain uniformly and appear more homogeneous.

Furthermore, Congo red dissolved in anhydrous glycerol does not stain injured granules, nor does it stain whole granules. After standing some time, however, the glycerol takes up water from the atmosphere and the injured granules begin to swell.[5]

The swollen portion of an injured granule loses its birefringence exactly like a granule gelatinized by heat. Here it should be pointed out that KATZ and HANSON (**50**) believe that wet grinding as employed by ZWIKKER and by them acts in a way different from dry grinding as employed by SPONSLER (**95**) and by ALSBERG and PERRY (**7**). KATZ and HANSON found that wet grinding does not destroy birefringence (black cross with nicols crossed) or the x-ray diagram. As we shall see, dry grinding does not destroy birefringence either, except in the injured portion. In starch ground wet, change

of pectin, nor does it serve similar functions. It has seemed best, therefore, to continue the practice of the writer and his co-workers in following TAYLOR and his co-workers (**102–106**) and SHERMAN and his co-workers by employing the term α-amylose for the insoluble fraction, and the term β-amylose for the soluble fraction. It is recognized that this practice is open to the objection that the Greek-letter prefixes have no essential connection with a stereochemical difference of the units composing the starch constituents, and that KATZ and WEIDINGER (**53**) have questioned the identity of TAYLOR'S substances with the correspondingly named ones of MEYER.

[5] According to FISCHER (**27**, p. 71), air-dry granules swell appreciably in a mixture of equal volumes of anhydrous glycerol and water.

in birefringence in the injured portion is not manifest because the injured portion swells and mostly disperses (*cf.* pp. 298–299). According to SPONSLER, sufficiently severe dry grinding destroys the x-ray diagram. According to KATZ and HANSON (*cf.* also **51**), wet grinding does not. This difference is probably due not to difference in mode of action of the two methods of grinding, but to the fact that wet grinding, as employed by KATZ and HANSON, did not disintegrate the granules nearly as completely as the very long dry grinding employed by SPONSLER. Starch subjected to a shearing pressure (*gleitendem Druck*) of 20,000 atmospheres loses its x-ray diagram (**65**).

The behavior of the swollen, injured part of a granule is similar to that of a granule swollen *in toto* by heating in water. Everything indicates that the swelling of injured granules is brought about by the same mechanism as gelatinization by heat. This suggests that neither gelatinization nor the alteration of the x-ray diagram brought about by gelatinization as described by KATZ and co-workers[6] is accompanied by chemical alteration of the molecule in the strict meaning of the word.

MEMBRANES

Many investigators (**65**) assume the existence of an outer, limiting membrane to explain some of the phenomena above described. In fact, there seems to be an outer, visible membrane, probably part of the protoplast which according to HANSON and KATZ (**34**), may be more or less lost in the process of preparation. According to ZWIKKER (**112,** pp. 61, 68), the plastid is removed in the preparation of starch. It is obviously a delicate structure. Perhaps it affects some of the physical properties of starch. Thus LACHELE (**55**) found that the granules of a commercial sample of wheat starch (Merck's), although neutral in reaction, became ruptured when swollen by boiling in water for 20 minutes, whereas a sample prepared by the method of RASK and ALSBERG (**75**), which employs no other reagents than 1 per cent. sodium chloride solution, water, alcohol, and ether, did not. Naturally, the plasticities of the two starch preparations were different. Possibly the difference in their behavior was due to the removal in the one case and the preservation in the other of a natural, outer membrane or coating. In any event, such an outer coating can be but little concerned in the phenomena under consideration. If it were responsible for them, then commercial starches which contain granules that have lost this membrane in whole or in part (*cf.* **34**) would contain an appreciable number of granules which are easily stained and disperse in water. As a matter of fact, such starches contain few such granules, and these are always plainly injured ones.

[6] See series of papers beginning in 1930: Abhandlungen zur physikalischen Chemie der Stärke und der Brotbereitung. In Zeitschrift für physikalische Chemie. Abteilung A.

THE MEMBRANE IN GELATINIZATION.—The view that natural granules have a strong outer memberane is based not upon seeing such a membrane with the microscope (*cf.* **64,** p. 149) in unaltered granules, but upon observations on gelatinized granules. These observations are of two sorts: the appearance of gelatinized starch, and the distribution of phosphorus chemically determined.

Under the microscope, gelatinized granules appear to be sacs with elastic walls enclosing sol or very dilute gel.[7] The existence of a membrane could be demonstrated with a dissecting microscope, the microneedles of which had been replaced by fine glass hooks. These hooks were made to puncture a sac at opposite poles. By gradually screwing the hooks apart, the sacs of potato starch could be stretched to about twice their length. If stretching is continued much beyond this point, the sac ruptures suddenly as if made of overstretched elastic. If the stretched sac is not ruptured and the strain is then relieved, it resumes its original shape. By means of a resistance wire, the paste was heated on the stage of the microscope and the manipulations repeated. It seemed a little easier to deform the warm granules, but the difference was not great. It was not possible to decide whether cold, gelatinized granules contain gel and hot ones sol (**32**).[8] There can be no doubt, then, that gelatinized granules are surrounded by an elastic, limiting membrane. It is this fact, principally, that has led to the inference, erroneous as we shall see, that this membrane preexists in natural granules.

PHOSPHORIC-ACID CONTENT OF MEMBRANE.—The membrane of gelatinized granules has been reported to contain phosphoric acid and has been assumed to consist of amylopectin which many investigators identify with the insoluble material of the granule. However, some starches consist wholly of β-amylose (*cf.* **38**).[9] The belief that amylopectin is the insoluble material of starch and

[7] That this is the structure of gelatinized starch is also shown by the fact that the consistency of starch paste depends upon the maintenance of this structure (*cf.* **3, 34**). Paste loses plasticity if the swollen granules are ruptured. Grinding in a pebble mill, for example, converts paste into a more or less viscous liquid, because the sacs collapse, thus no longer occupying nearly all the space of the system (**7**).

[8] Analogous observations were recorded by HESS and RABINOWITSCH (**39**), who examined granules that had first been gelatinized in water and then put into dilute alcohol. This may account for some of the minor differences between their observations and those of GRIFFING.

[9] In this connection, it is worthy of note that in the only starch (rice) in which the ash of the glutinous and non-glutinous varieties was compared, the non-glutinous variety had, on the average, more phosphoric acid and silicate than the glutinous variety. But some samples of the latter contained more of both than some samples of the former (**101**). The glutinous variety moreover, had the greater swelling power and yielded the more viscous pastes in spite of its generally lesser phosphoric acid content, a fact which does not support the view held by SAMEC and others that viscosity depends principally upon the phosphoric acid-containing fraction.

that it forms the membrane of gelatinized granules has led many investigators[10] to assume that the peripheral zone of ungelatinized granules also consists principally of α-amylose (amylopectin). A difference in the phosphoric acid content of large and small granules of the same species has been advanced as evidence for this view. The reasoning is as follows: If α-amylose is localized principally at the surface of granules, small granules of the same sample should contain a larger percentage of phosphoric acid than large ones, assuming the thickness of the membrane to be about the same in all granules, because the ratio of surface to mass is greater in small than in large granules. Small ones have a larger surface, relatively, and therefore a larger percentage of their mass should be in the membrane, if this is not thinner than that of large granules. SCHOEN (**86**), however, would attribute any greater phosphoric acid content of small grains to sorption on their greater surface. HINES made an investigation under the writer's direction upon cassava starch, separated into fractions of different granule size by air flotation in an air classifier, and failed to find any correlation between granule size and phosphoric acid content (**1**). It is therefore clear that there are starches for which no conclusion regarding the distribution of amylopectin can be drawn from their phosphoric acid content.

Furthermore, evidence controverting the view that small granules contain more phosphorus than large ones is to be found in the behavior of granules when boiled in water. If α-amylose (amylopectin) were localized at the periphery, one would expect those natural granules to have the thickest and strongest membranes that have the greatest phosphoric-acid content. One would expect such granules when boiled to hold together better than granules with a lesser phosphoric-acid content. As a matter of fact, there is no such relationship between phosphoric-acid content and resistance to boiling. Potato starch with a fairly high phosphoric-acid content disintegrates rapidly, whereas tapioca, also a root starch but with a much lesser phosphoric-acid content (**106**), does not (**31**).

Moreover, MAQUENNE and ROUX (**60**), who first estimated the amylopectin content of granules, believed that it is intimately mixed with the other constituents. SCHOCH (**85**), in his study of the distribution of phosphoric acid, reached the conclusion that phosphoric acid is quite irregularly distributed through the natural granule.

[10] For example, (**66**, p. 216). LING and NANJI (**57**), however, hold a different view. They suggest "... that 'amylose' as it exists in starch granules is present in more than one physical state and possibly in different degrees of hydration ... about 25% of the 'amylose' exists as a crystalloidal phase in the form of spherites constituting a core round the hilum of the granule. This portion is readily extracted by water or by dilute alkali. The remainder, which presents a colloidal phase, is dispersed uniformly in the amylopectin layers. It appears to exist either as a solid solution or to be so strongly adsorbed on the amylopectin as to resist extraction."

THE MEMBRANE AN ARTIFACT.—In summary, it may be said that there is no doubt that gelatinized granules are surrounded by a fairly strong membrane. The evidence that in ungelatinized granules amylopectin is concentrated in a peripheral membrane is doubtful. This, together with the absence from natural granules of a visible membrane of the thickness and toughness of the membrane of gelatinized granules, leaves the question of the existence of such a membrane open.

But the facts regarding swelling, shrinking, and staining, above arrayed, cannot be reconciled with the assumption that natural granules are surrounded by a membrane which is impermeable to many dyes, and to the starch substance itself. If the resistance of intact granules to staining and to more than moderate swelling and their insolubility in cold water were due merely to the existence of such a simple membrane, then in an injured granule with a locally ruptured membrane staining should be general throughout the granule and swelling in cold water should be general also. As we have seen, none of these phenomena occur. They are strictly localized to the site of the injury. As we shall see later, the birefringence of granules, as well as their solubility, is affected in an analogous way.

It is conceivable that the granule is surrounded by a membrane so rigid that it prevents mechanical disorganization except at the site of the injury where the membrane is broken. This is, however, improbable, because if there were a rigid membrane permeable to water this should either prevent shrinking or else one should be able to observe a separation of the shrunken granule substance away from the rigid membrane. It is improbable that a rigid membrane and the granule substance both shrink to exactly the same degree. Certainly in a dried granule no separation between membrane and granule substance is to be seen. If there is a limiting membrane which is elastic, like the membrane surrounding a gelatinized granule, it could hardly account for the strict localization of the swelling to the site of injury.

Some investigators have assumed the existence of a simple system of membranes represented perhaps by the laminae or rings. If there is such a system, the laminae are hardly a part of it, for unlaminated starch swells like ordinary starch (**12**). Moreover, a granule broken in two by a fracture passing through the hilum has all of its rings opened up. Any simple membrane system that might be present would be opened up widely so as to permit extensive, if not complete, swelling and staining. As we have seen, these phenomena are strictly limited to the immediate region of the fracture. Certainly, a series of concentric membrane rings could not explain the facts. Nor could a series of radial membranes. Nothing short of a system consisting of concentric, hollow spheres, divided by many radially arranged transverse planes could explain the behavior of fractured granules.

KATZ and RIENTSMA (**52**) have, indeed, suggested this very possibility. KATZ (**46**) subsequently elaborated this idea as follows: "There might be a binding substance in a swelling substasce which binds packages of micellae together to larger, probably, but still submicroscopical units. In grown products, tissues, fibers, starch granules, this binding substance might take the form of membranes lying between the micellae or between small bundles of micellae."

Subsequently, HANSON and KATZ (**34**) obtained support for this view by the examination of Lintner starch,[11] which easily fragments into concentric rings composed of separate rectangular blocks regularly arranged radially. These blocks they believe to be composed of bundles of parallel crystals, for they are normally birefringent. They believe further that the blocks are held together in natural starch by a cementing substance, possibly amylopectin (α-amylose), which is dissolved out by the acid in the process of lintnerization. Lintner starch does not swell in cold water, but disperses to a clear solution in warm water. It does not form paste because the cementing substance which holds the granule together and swells in the process of gelatinization has been removed. It is the cementing substance, they suggest, that forms the membrane system of the natural granule.

It is altogether likely that the view that there is intermicellar substance is correct,[12] but it may be doubted that it serves as a membrane system, for its presence does not seem capable of explaining the swelling of injured natural granules. Why should the cement substance swell so little in the cold in an intact granule and so greatly in an injured one? Obviously, some additional factor is involved.

On the other hand, if the natural starch granule swells greatly in water after it has been broken up by mechanical means, why does it not swell when it is broken up into blocks by Lintner's treatment? It must be either that the prolonged acid treatment so greatly modifies the granule's substance that its swelling power and solubility are radically altered, or else that, of the substances of which the granule is composed, only one, the cementing substance, swells and disperses cold. The Lintner process may remove this cementing substance.[13] In that event, the other material, composing the blocks of Lintner starch, neither swells nor disperses in water, except when warmed. Thus we might explain the properties of Lintner starch; but we would not explain why natural intact granules which contain the swelling substance do not swell greatly unless warmed, whereas the same granules, still containing the swelling substance, swell greatly at once when injured.

[11] Starch subjected for a long time to 7.5 per cent. hydrochloric acid.

[12] LOSKIT (**58**, p. 154) concluded from the optical properties of starch that it must be a mixture of colloidal and crystalline particles.

[13] Commercial soluble starch and the artificial starch granules of MAQUENNE and ROUX do not yield amylopectin (**33**).

We are thus forced to the conclusion that such membrane as natural granules may possess is not identical with the limiting membrane of heat-gelatinized granules. How is this to be explained? Obviously, by the assumption that the membrane surrounding heat-gelatinized granules is an artifact. ZWIKKER (**112**) has demonstrated that this is true. He has correctly described the origin of the membrane during gelatinization thus: "The outer parts of the grain become distended; they detach themselves at the same time from the central mass or remain adherent to it. In one case as in the other, a cavity is formed in the interior of the grain. The warm water or any other liquid that may have been used to make the starch swell is thus aspirated so to say into the interior. In passing through this wall it [the liquid] removes from it [the wall] and carries along with it [the liquid] the dispersible substances; in consequence, when the gelatinization has become complete, the membrane is only composed of the elements that are soluble with difficulty derived from the different parts of the grain and especially from the most resistant layers; all these elements are found agglutinated into a homogeneous mass."[14]

That granules in process of being gelatinized by heat do show a central cavity which gradually enlarges, has also been observed by the writer and by many others (**64**).[15] Very commonly, this cavity is at first stellate, due to the radial structure of the granule. The observations of BAKHUYZEN (**12**) upon wheat starch devoid of laminae support the view of ZWIKKER. There is, therefore, no necessity to assume the pre-existence in natural starch of a rigid, limiting, outer membrane. The gelatinized granule's membrane is clearly an artifact.

On the assumption that natural granules possess no limiting membrane, the behavior of compound granules, when gelatinized, becomes understandable. After they have fallen apart into their component granules each of the latter forms its own limiting membrane when gelatinized, exactly like a simple granule. And this occurs even when no membrane separating the component granules is visible in the original unbroken compound granule.

Furthermore, occasionally two granules that lie close to one another in the same protoplast become enveloped by a capsule of starch substance which shuts them off completely from their surroundings. MEYER terms them complex granules. When they are gelatinized by heating in water the whole complex is connected into a sphere with only one outer limiting membrane (**70**, pp. 210, 224). If the two granules had each its own outer membrane, one would expect the complex granule to be changed into a large sac surrounding two smaller sacs—one for each of the component granules. Their

[14] The writer's translation from the French original.

[15] A drawing of a granule in this state is given by HANSON and KATZ (**34**).

behavior when gelatinized is easy to explain on ZWIKKER's hypothesis but difficult on any other.

UNLAMINATED STARCH

The facts presented seem to warrant the conclusion that it is not a membrane system that keeps the natural granule from swelling beyond definite limits. If this is so, what is the mechanism that is responsible for this phenomenon? The hypothesis advanced in this paper presents such a mechanism: it is the radial arrangement of the crystallites and starch chains. To make clear how this simple physical arrangement explains the facts, it is best first to consider granules without laminations or rings. This is justifiable because the occurrence of laminations is accidental, rather than essential, otherwise they would never be lacking in any sort of starch. But they are occasionally missing from many sorts of starch and their absence is the rule in the root starch of *Zingiber officinale* (**70**, p. 17), in the starch of the yellow turnip, and the seed of *Ruellia pavale* (**109**). MEYER (**64**) attributed their formation to the alternation of day and night; moreover, BAKHUYZEN (**12**) failed to find them in seed starch from wheat grown in an environment from which this sort of periodicity had been eliminated—that is, an environment nearly constant with respect to illumination, temperature, and humidity.

BAKHUYZEN's unlaminated wheat starch has very definite radial structure. In it, the crystallites may be seen to reach from the periphery to the hilum. When these granules were carefully heated in water so that they swelled slowly, he found that they could be seen to be composed of very refractive radial needles attached to the refractive border of the granule. These needles are tapered toward the center, resembling pyramids with a base 2 to 3 microns in diameter and a length of 17 to 20 microns, which is about 45 per cent. of the total diameter of the granule. In such unheated, unlaminated starch, probably all of the needles (crystallites) stretch from the border to the hilum.

THE STARCH ELEMENTS

X-ray analysis has definitely established that the starch granule has crystalline structure. Most probably the ultimate starch elements are long units (**45, 98**, p. 75).[16] It is not yet possible to say just what is their form, but it is probably not a straight chain, for since the elements of the chain are apparently held together by α-glucosidic linkages, a straight-chain structure is not possible (**37**). Perhaps starch chains are spirally wound (**65**),[17] like the thread on the end of a bolt that receives the nut, or have a zigzag (**65**) shape

[16] See, however, (**19**).

[17] It is interesting to note that spirally twisted granules have been reported by NÄGELI (**70**, p. 31).

like the bellows of an accordion, or a meander (**99**) form, being fluted more or less like a sheet of corrugated roofing iron. In any event, the long chains seem to be folded or crumpled in some manner as yet unknown, whereby the molecule is shortened—compressed as it were—in the direction of its long axis, for a starch molecule consists of 8 to 10 times as many glucose residues as a cellulose molecule of the same length (**99,** p. 828). Perhaps the chains are crumpled and the long axis of the molecule shortened because certain of the side groups of the macromolecule attract each other strongly. This would tend to bend the long axis and shorten the molecule. It is possible the chain is also forked or branched (**66, 99**). Units of this type would not pack as closely as straighter chains, for example, those of cellulose. This may be one of the reasons why starch has a looser structure than cellulose, is more easily penetrated by solvents (**99**), and is more readily subject to mechanical influences (**45, 95**).

In these long units, the atoms are linked together by primary valences. It is probable that van-der-Waal or cohesive forces hold these macromolecules together in more or less parallel arrangement. Whether in this way the macromolecules are held together in separate and discrete bundles to form micelles is not clear. At present, the view seems to be gaining ground that the micelle, in general, is not always and necessarily a separate and discrete unit and that fibrous structures like cellulose need not have a completely discontinuous framework. NEALE (**72, 89**) has suggested that in certain cases, at least, the structure is not "micellar" or discontinuous, but that, though imperfectly crystalline, it is continuous except for random breaks in the primary valence chains and that though continuous it is slightly imperfect. TAYLOR and SCHOCH (**105**) have suggested the presence in starch "of more or less completely esterified tri-basic phosphoric acid, either with hydroxyl groups on one chain or bridging between two chains of glucose anhydrides."

GRANULE STRUCTURE

Everything indicates that the chains, or the bundles (micelles) into which the chains are perhaps grouped, are arranged radially.[18] Of course, the radial granule structure visible with the microscope is a coarser one than that of the macromolecules or even the micelles. Each of the visible radial needles must be regarded as a crystallite composed of many chain molecules or of many micelles of such molecules.[19]

A granule thus constructed would be strongest in a radial direction. The primary valence bonds which hold the glucose residues into a chain would

[18] This is similar to the anatomical structure proposed by MEYER (**64**). The bundles would correspond to his trichites, but he assumed the trichites to be forked.

[19] Whether the macromolecules are arranged parallel in the crystals has not yet been proved by x-ray analysis, but is probable according to MEYER, HOPFF, and MARK (**65**).

make for greater strength lengthwise of the chain than would the secondary valences which hold the chains to one another laterally (**94**). In consequence, there is a tendency for granules to rift radially when subjected to mechanical strain. The force required need not be very great; as we have seen, mere thorough drying may cause radial cracks to appear. This fragility need not be taken to mean that the structure of the granule is everywhere discontinuous from micelle to micelle or that chemical cross linkages between chains are necessarily absent.

Does this suggested structure explain the shrinking and limited swelling of natural granules?

Let us first consider the shrinking of granules when dried. If the starch units are long chains, then when they swell they must increase in diameter much more crosswise than lengthwise, if indeed they lengthen longitudinally at all (**96**).

And conversely, they must diminish more in their transverse diameter than longitudinally when they shrink. This seems to be the usual behavior of fibers. The diameter of the wool fiber becomes about 18 per cent. greater when it is wetted, but only 1 per cent. longer (**8**). Therefore, it is difficult to explain the very considerable shrinkage of starch granules when they are dried (**78**), for the radial arrangement and the swelling and shrinking of long chains in their shorter diameter should result in swelling or shrinking in a tangential direction. As a consequence, the shrinking granule should break up into wedge-shaped pieces. This is actually the behavior of inulin spheroliths (**27**) and of the crystalline disks of amylodextrin (**69**). It is probably also a factor in the radial rifting of dried starch already noted. Since, however, the starch granule swells and shrinks in a radial as well as a tangential direction and increases or decreases its diameter, we must assume either that the starch chains do not behave like wool fibers or else that for some reason they are prevented from so behaving.

This difficulty disappears if we assume the starch chains in the natural moist granule to be already stretched mechanically by their own swelling pressure. As we have seen, according to STAUDINGER and EILERS (**99**), the starch chain is shortened in some way, as compared with cellulose, a conclusion based in large part upon viscosity determinations of starch solutions.[20] In solution, the macromolecule is under little or no mechanical strain. It is, therefore, presumably much crumpled so that its long axis is much shortened. It may, however, not be maximally contracted. There is no basis for knowing whether starch macromolecules have the same length in solution as in the natural starch granule.

[20] This interpretation of the significance of viscosity measurements has, however, been questioned (**18, 62**).

It is obvious that at maximum contraction the region over which water or other solvent might associate itself with the macromolecule is shorter than when the macromolecule is more or less extended. In the contracted state, secondary valences are in part neutralized by the very intramolecular attraction that shortens the chain. Moisture sorbed on natural starch granules is under quite enormous pressure (77) and we are, therefore, justified in assuming that water exerts very great force in attempting to associate itself with starch. In doing so, water would endeavor to make as much room for itself along the length of the chain as possible. To this extent, the forces crumpling the chain, such, perhaps, as the intramolecular attraction of side chains for one another, would be counteracted and weakened. The chain would tend to straighten. It is, therefore, probable that starch molecules, while shorter than cellulose molecules though with the same number of glucose residues, are still, as STAUDINGER and EILERS believe, quite long chains when in solution. In this condition, they are neither fully contracted nor fully extended. When a starch solution is dried to a film, there may well be further contraction of the macromolecules.

If these conceptions correspond to realities, it follows that, in natural moisture-containing starch granules, the chains are extended to the degree that associated water has stretched them. But their state of extension is probably not the same as in a starch solution. Presumably, as in the case of wool, the association of water tends to thicken the diameter of the chain. We would, then, have two factors at work in opposite directions: lengthening of the chain which would tend to make it thinner, and the pressure of associated water which would tend to make it thicker.

When a cellulose or a wool fibril sorbs water, it increases greatly in its transverse axis and but slightly along its longitudinal axis. Such a fibril is free to expand in all directions except inward where it is hindered by other fibers also swelling similarly, and, therefore, increases greatly in diameter. A starch fibril is in a quite different position. Because of the radial arrangement of the granule, it is surrounded on all sides of its long axis by other fibrils also endeavoring to swell in a direction transverse to their long axes. Every macromolecule is prevented from associating itself with water to its maximum capacity because it is hampered mechanically by its position with reference to its neighboring macromolecules. That is why the intact starch granule is strictly limited in its capacity to swell despite the fact that its substance has unlimited capacity to swell and disperse. No limiting membrane is necessary to account for the phenomenon.

According to our hypothesis, the macromolecules in moist natural starch are perhaps unable to associate themselves with as much water as is possible when they are free in solution. Nevertheless they may be more extended along their long axis when *in situ* in the moist granule than when free in solution.

for in the granule the macromolecules squeeze one another laterally; they subject one another to strain. In consequence, they tend to push one another apart and, since this is possible only to a slight degree, they flatten and lengthen one another.

The possibilities for such lengthening are appreciable, since the chains tend, if not subjected to strain, to assume a very crumpled form. The dry granule, when moistened, tends to swell and its thickness is a function of the moisture content. Any condition such as heating that changes the moisture content, *i.e.*, the association or sorption of water, changes the size of the granule; it shrinks or swells. In the natural moisture-containing granule, the chains are already stretched to some degree due to their own swelling pressure. That is why the granule shrinks when dried and swells again when moistened.

It is also the reason why the moist granule does not swell beyond a certain size. It could do so only if there were room for the macromolecules (or micelles) to thicken more; but there is no room because they are surrounded on all sides, except at the peripheral end, by other macromolecules or micelles which are also trying to thicken. Swelling, therefore, stops when equilibrium is reached between the force making for thickening and the resistance to thickening.

This equilibrium may be disturbed by increasing the kinetic energy of water as by heat. Molecular agitation which is associated directly with the coefficient of thermal expansion would be less lengthwise than crosswise of the starch chains (*cf.* **97**) and therefore granules tend to swell more in warm than in cold water. Greater swelling in warm water can take place only if the diameter of the granule enlarges. Since the macromolecules (or micelles) swell mostly laterally, this can take place only in the following ways: (1) The macromolecules straighten their long axes by water sorption and pressure against one another. (2) The macromolecules push one another apart, though still cohering. In consequence, the diameter of the granule increases greatly so that spherical granules are converted from solid spheres into hollow globes. (3) The granule is disorganized and more or less dispersed. (4) Or, there is a combination of these phenomena.

The first phenomenon, the straightening of the macromolecules accompanied by water sorption, probably occurs in the early stages of gelatinization and may perhaps account for changes in the x-ray diagram.

The second phenomenon, the conversion of a solid spherical granule into a hollow sphere, may actually be observed when the granule is slowly heated in water. It swells and, as we have seen, a cavity develops at the hilum, because the degree to which the chains may lengthen must be strictly limited. The granule cannot swell greatly and still retain its solid form. Spherical granules become hollow spheres. Other granules swell in a way determined

by their form and the fact that swelling develops pressure tangentially. Thus, it is well known that eccentric granules (*Solanum, Phajus,* cassava, *et al.*) swell relatively more in breadth than in length.

KATZ and HANSON (**50**) have assumed that the swelling of solid granules to hollow spheres is a unique phenomenon, and that the only possible way to explain it is to assume some sort of growth structure in the native granule. They have not themselves determined the nature of this structure resulting from growth. Protein crystals, however, also swell with the formation of a vacuole in the interior, and may be made artificially. SCHIMPER (**83**) attributed the phenomenon to the greater solubility of the material in the interior of the crystal, and this is the explanation NÄGELI had offered earlier for the analogous phenomenon in starch. However, the explanation offered by ZWIKKER and by BAKHUYZEN for starch seems equally applicable to protein crystals, if one assumes that the outer layers swell soonest and tend to draw away from the center, thus creating a vacuole. It is then unnecessary to assume, as did SCHIMPER, that these crystals consist of more than one protein, which would not be in accord with present-day knowledge. Nor is it in accord with the fact that WYCKOFF and co-workers have obtained x-ray diagrams from protein crystals. Some protein crystals behave so much like starch with respect to swelling and solubility that if one has a "growth structure" then they both have. This "growth structure" is the arrangement of their molecules in the crystal. The hypothesis here presented, should it be supported by further experimental evidence, offers a simple and reasonable explanation of the phenomena.

With heating, the granule swells greatly. The second phenomenon, disintegration, has then occurred; the granule has been converted into a sac filled with dispersed starch substance; and we have the third phenomenon. The degree to which disorganization occurs is a function of the amount of water available, as has been shown by MEYER (**63**).

If this hypothesis should ultimately prove to be well founded, then analogies with rubber at once come to mind. The moist starch granule is elastic; it changes its shape under pressure and returns to its original form without the slightest change in its microscopic structure. Dry granules, on the contrary, are inelastic and brittle. Rubber, too, under certain conditions, loses its elasticity. Moreover, it is well known that rubber exhibits the x-ray diagram of amorphous substances unless it be stretched, in which case it exhibits a fiber x-ray diagram. When it is quite anhydrous, starch, like unstretched rubber, exhibits no x-ray diagram, or rather it exhibits the diagram of an amorphous substance (**45**). It acquires an x-ray diagram only if it is permitted to sorb moisture, but the diagram is not that of a fiber. It is more like that of a crystalline powder. According to our hypothesis, we may consider the macromolecule of a natural undried granule as already

partially stretched by associated water, very small quantities of which are capable of stretching the chains to give them an orderly arrangement sufficient for an x-ray diagram.

Hitherto it has not been possible to obtain a fiber x-ray diagram from starch. Neither spinning threads from starch solutions nor pressing preparations was effective (**65**). For natural starch granules, a fiber x-ray diagram is not to be expected. For a fiber diagram, it is necessary that the chain molecules of the material be oriented so that their long axes for the most part are parallel. In a powder consisting of starch granules, this is not the case. Nor would a single starch granule yield a fiber diagram, if it were possible to obtain an x-ray diagram with it. Although the macromolecules are probably parallel in the individual crystallites, the crystallites themselves are not parallel to one another. Their arrangement is radial. Therefore, although in the moist granule the macromolecules may be regarded as more or less stretched by their own swelling pressure, they cannot yield a fiber diagram.

NÁRAY-SZABÓ (**71**, p. 299) and KATZ and DERKSEN (**48**) have studied the loss of the x-ray diagram in quite anhydrous starch. KATZ (**45**, pp. 3633–3635) has also described a similar behavior for gelatin and agar, and has offered the following alternative explanations: One of them is that the substance when dried changes into another modification, another phase within the meaning of the phase rule. This means that the material, *e.g.*, agar gel, is built up of crystallites which contain water of constitution. The other alternative is that in drying no change of phase occurs (within the meaning of the phase rule) involving a discontinuous modification of individual units, but rather there is a continuous change involving a gradual loss of regularity in the lattice. KATZ makes no choice between these alternatives in the case of gelatin and of agar, but in the case of starch he is inclined to believe that there may be something like true water of crystallization. Perhaps he favors this view because all starch preparations (soluble starch, amylodextrin, etc.) exhibit the same phenomenon (**45**). What is meant by "water of crystallization" is, of course, a matter of definition. In the case of starch, the phenomenon is probably not exactly the same as in the case of the efflorescence of an inorganic salt in which the water is held in stoichiometric proportions. Nor does the phenomenon seem to be exactly the same as that which occurs in the case of certain protein crystals which show no x-ray diagram unless great care is taken to prevent efflorescence (**111**). In many soluble proteins, this process does not seem to be reversible except through recrystallization; in starch it is. One thing seems certain: water is held in starch very firmly. FISCHER (**27**) found that starch granules treated with a dilute solution of cobaltous chloride ceased to be colored rose red long before they became anhydrous. On the contrary, they turned in-

tense blue, showing that the water of partially dried starch is held so firmly it is not available to the cobaltous chloride.

It is also difficult to understand why the birefringence of the starch granules, as made manifest by the black cross, should persist in anhydrous starch, as we shall see it does, if the crystals weather. The black cross appears because the crystallites are approximately parallel, an arrangement one would hardly expect to persist after weathering. It is therefore doubtful that the behavior of starch when sharply dried is due to exactly the same phenomenon as the weathering of a crystal losing water of crystallization.

Furthermore, it is difficult to explain as a case of efflorescence (**95**) the loss of the x-ray diagram when natural starch is thoroughly dry-ground in a pebble mill. Our hypothesis, however, offers a basis for a common explanation for both phenomena. This hypothesis assumes that, if there are no effective contravailing forces, starch molecules tend to contract along their long axes, to crumple up. The evidence at hand and already presented does not prove this innate tendency, but it does make our assumption at least reasonable. In the natural moisture-containing granule, the macromolecules are not fully crumpled, because with the adsorbed moisture they occupy too much space for them to contract fully. They are more or less stretched. As the granule is dried, the chains become thinner, occupy less space and in consequence are able to contract further. In the process, they lose their orderly arrangement sufficiently to sacrifice their x-ray diagram. Perhaps the disorderly arrangement arises because contraction is not uniform from macromolecule to macromolecule, and therefore the diffracting groups of individual molecules change their positions with reference to one another from macromolecule to macromolecule. One need not imagine a more extensive disorganization. This idea comes to pretty much the same thing as the second of KATZ'S alternatives. In the moist natural granule, the macromolecules are prevented from crumpling because they can shorten only by thickening, and being associated with water, there is no space for them to shorten. If, however, the radial, parallel structure is sufficiently broken up by mechanical disintegration of the granule, there is no longer the impediment to contraction that exists in the intact granule, even in the presence of some moisture. A large number of the macromolecules are then free and the x-ray diagram is more or less lost.[21]

Our hypothesis is also consistent with the changes in x-ray diagram that occur in starch when it is made to swell. It is well known that stretching such fibers as wool or hair changes their x-ray pattern—presumably because

[21] It would be interesting to determine whether dry grinding has an analogous effect upon the x-ray diagram of gelatin. It is possible, for ALSBERG and GRIFFING (**5**) found it to have a marked effect upon solubility and gel formation. It would also be interesting to examine gelatin films made by drying sols and gels. FIELD (**25**) has shown that the former are not birefringent as are the latter.

the chain molecules of which they are composed are straightened and lengthened (**9**).[22] KATZ and co-workers (**34, 45–53**) have found that starch, when made to swell in water by heating, may undergo alteration of its x-ray diagram, and that in certain cases this alteration is reversible. They suggest that the change is due to the formation of different modifications of starch. The phenomenon might, however, be explained according to our hypothesis as the consequence of lengthening or shortening of the chains.

The hypothesis suggested would also explain the hysteresis which, as we have seen, hinders dried granules from returning to their original size when moistened. According to this hypothesis, the chain macromolecules shorten or crumple as the granule loses water, else the granule would not shrink. With the removal of water, the macromolecules are brought closer together than they have ever been, for it must be remembered that starch is formed in a *milieu*, the cytoplasm, which has a high water content. Unless starch is artificially dried, it always contains a fair amount of moisture. With the removal of moisture and the moving closer together of the contracted but thickening chain molecules, linkages through secondary valence are perhaps formed between side groups that have never before been united. As already indicated, this might also lead to distortion of the lattice and loss of the x-ray diagram. SPEAKMAN (**90, 91**) has assumed something of the same sort to occur in wool, heated to higher than ordinary temperature, a treatment which reduces the capacity of the fiber to sorb water hygroscopically. The black cross still remains in the anhydrous granule, for probably the contraction of the long axis of macromolecules, even though it distorts the lattice, does not greatly disarrange the parallel arrangement of the macromolecules or distort the crystallites upon which the birefringence depends.

When a granule in this state is moistened, it is necessary that these newly established linkages be broken up, if it is again to swell to its former state. Obviously, this would take time, and would occur imperfectly. Consequently, the granule would swell slowly and not fully to its original size.

We now see why injury to the granule enables it to swell at the site of the injury. The injury separates some of the macromolecules or micelles so that they are no longer surrounded on all sides by other macromolecules or micelles. They associate themselves to their maximum capacity with water. Again no limiting membrane is necessary to account for the phenomena, which are due merely to the fact that through the site of the injury the orderly packing of the chains has been disarranged. ITALLIE (**42**) has made an analogous suggestion but he also assumes the existence of a limiting membrane. It is plain why the degree of swelling in cold water depends upon the completeness of the disruption of the granules. Our hypothesis also

[22] This view was criticized by KATZ (9, pp. 207–209), to whom ASTBURY (9, pp. 210–211) replied.

explains the observation of KATZ and HANSON (**50**) that injured granules change the character of their x-ray diagram at a temperature 10° to 20° C. lower than that at which uninjured granules undergo this change, and also why injury wipes out to a large extent differences in this temperature which are shown by different species of starch in the uninjured state.

GROWTH

Structure of the type here suggested is consistent with what is known of the manner of growth of starch grains. If starch grains possessed an outer limiting membrane permeable only to substances of comparatively low molecular weight, it would be difficult to understand how small granules grow into large ones. There is firstly the difficulty of imagining how a limiting membrane of the type demanded by the properties of the natural granule stretches and enlarges simultaneously with the growth of the granule. Certainly, the long molecules of starch, if formed in the protoplasm outside, would find it difficult to pass through such a membrane. It would, therefore, be necessary to assume that mono- or disaccharide passes from the protoplasm across the membrane into the granule there to be united into long chains and oriented radially. This would demand that the directive forces residing in the cytoplasm act across the membrane, or else that cytoplasm itself extends through the membrane into the granule. For such assumptions, there does not as yet seem to be evidence. If, however, there is no limiting membrane of the sort under discussion, but the cytoplasm—perhaps in a condensed form—lies in contact with the growing granule, then it is simpler to picture the growth of a granule much as SPONSLER (**92, 93, 94**) explains the formation of a cellulose fiber in the cell wall or FARR and ECKERSON (**22**) explain the formation of the cotton fiber.

A granule might grow through lengthening of the starch chains by the condensation of a glucose or maltose residue at the peripheral end of the chain, so that the chains grow longer. Or perhaps the method of growth is more like that described for cotton fibrils by FARR and ECKERSON. In that event, small starch crystallites are formed in the cytoplasm surrounding the small granule. These then arrange themselves radially around the granule and become attached to or inserted between the chains of particles already arranged radially in the growing granule.

As the granule grows, the chains must lie less closely packed at their peripheral ends. This leaves room between them for the formation of new chains by the same process by which the original chains were formed; and these new chains are also radially oriented between the outer ends of the older chains. Thus, crystallites are built up which are irregularly conical with their bases lying at the periphery of the granule. Such a form is clearly to be seen

in unlaminated starch in which the crystallites extend from the periphery to the hilum (**12**), a phenomenon which can be explained only on the assumption that new starch substance is added at the base of the needles.

This picture of the growth of starch granules is not the same as that suggested by ZWIKKER (**112**), but it is not inconsistent with it. ZWIKKER suggests that the leucoplast secretes a concentrated colloidal solution of amylose and amylopectin. Now starch lowers the surface tension of water, so that during an interruption of the activity of the leucoplast there is at first a concentration of the substance with the greatest effect on surface tension (*e.g.*, a potassium salt of amylopectin) at the surface accompanied by its separation or precipitation. When the leucoplast again becomes active, this layer remains unaffected and the process is repeated. ZWIKKER, who wrote in 1921 when the x-ray analysis of organized structures had not been developed as it is today, has nothing to say regarding the formation of colloidal amylose or amylopectin. Now that we know something regarding the form of the starch unit, we can go a step farther and extend the picture to the manner of formation of the chains. It is entirely possible that surface forces play a rôle, as ZWIKKER suggests, in determining the parallel arrangement of the chains in building up particles of the type observed in the case of cellulose fibers by FARR and ECKERSON.

If this is in fact the mechanism by which starch granules grow, there is no reason to believe that the chains or macromolecules of which the crystallites are composed are all of the same length. Indeed, the chances are greatly against it. It is probable that starch, like many other materials (*cf.* **98**, pp. 109, 112–113; also **65**), is composed of macromolecules of varying length. NEALE (**72**) has presented evidence that this is true for the cellulose fiber. There is much evidence that this is in fact the case for starch. Thus FOUARD (**29**; *cf.* **79, 81**) has reported that solutions of demineralized starch can be fractionated by dialysis through collodion membranes of different permeability. The occurrence of chains of different lengths is also consistent with the views of HANSON and KATZ regarding the structure of the granule. Probably the arrangement of the molecules in starch granules resulting from the manner of growth above suggested causes imperfect packing and may be a factor leading to the loose structure of granules already discussed.

Whether the elements in the unlaminated starch of BAKHUYZEN extend, as they appear to do under the microscope, from the hilum to the periphery, it is not possible to decide from existing evidence. Some of the starch elements may be as long as this and consist of macromolecules which are joined together by van-der-Waal forces to form quite long fibers. This is well known to occur in the case of soaps, and has been suggested for starch (**10**). Moreover, it should be noted that SPONSLER believes that the diffraction data obtained from x-ray analysis of cellulose may be accounted for upon the basis of chains of cellulose-unit cells of indefinite length.

The laminated starch granule

Bakhuyzen correlates the occurrence of the large needles in starch from wheat plants grown under constant conditions with the absence of lamination or rings. In field-grown wheat seed he finds the needles or trichites no larger than 5 microns, or about 10 per cent. of the diameter of the wheat-starch grain. Ten to 20 or more rings can be counted. Larger needles may also be observed. If such starch is carefully warmed to swelling temperature, the laminae may be observed to consist of small particles. Particles in successive rings may often be seen to be oriented along a straight radial axis, suggesting that such a string of particles was formed from a single needle which had been fragmented by heating. The parts of the needles which correspond to the non-refractive rings or laminae, however, remain.

Much evidence indicates that laminated starch is built up of bundles of parallel chains combined to form crystallites of a length about equal to the width of the laminae. If starch granules develop in the manner described for cellulose fibers by Farr and Eckerson (**22**), as we have suggested, then it is quite probable that in some starches the thickness of laminae is determined by the length of the starch crystallite formed in the cytoplasm. Certainly in some, the breadth of the laminae is of the same order of magnitude (1 to 2 microns) as the longest diameter of the cellulose particle of Farr and Eckerson (*cf.* also **23**). In eccentric starches, the laminae at the pole nearer the hilum may be very narrow, indeed. If the growth of the starch granule takes the course suggested by Farr and Eckerson for cellulose, then any periodicity which interrupted the process must of necessity lead to the deposition of starch substance in layers or laminae.

This view, based on the observations of Bakhuyzen and others, is in harmony with the observations of Hanson and Katz (**34**) who, as we have seen, describe the granule as consisting of short elements radially arranged and embedded in an amorphous matrix.

Solubility

It has been known since the first part of the nineteenth century that, while starch granules do not dissolve in cold water, a starch granule which has been injured by cracking, chipping, or grinding disperses more or less[23] in water without warming. It has furthermore been shown that the degree to which the granule disperses depends upon the extent to which it has been disintegrated mechanically, for with very thorough grinding all the β-amylose can be dispersed and extracted from corn starch by cold water (**85, 102**). Those who, like Matthews and Lot (**61**), believe that only a minor part of the substance of injured granules is soluble in cold water are cer-

[23] Literature to 1895 in Meyer (**64**, p. 18); to 1910 in Reichert (**76**). Literature not cited in the above volumes (**3, 5, 16, 21, 28, 35, 36, 41, 54, 95, 102, 108, 112**).

tainly mistaken. This solubility in cold water which results from injury to the granule is not due to alteration of the starch substance by frictional heat engendered in disrupting the granule (**41, 43**). Nor is it at all likely, as SAMEC (**80**) states, that grinding as such breaks up the starch complex into smaller aggregates, which disperse readily; the manipulation necessary to injure a granule so that some of its substance disperses is far too slight. All that is necessary is to put a little dry starch on a microscope slide, cover it with a glass cover slip, and press gently upon it for a moment, at the same time moving the cover slip a trifle sideways.[24] Moreover, as we have seen (p. 309), merely drying sharply causes radial rifts which render some of the granule soluble.

Furthermore the colloidal solutions obtained from potato starch ground in a pebble mill do not behave as though the starch had been broken up into smaller complexes. The solutions are strongly opalescent and change rapidly. The concentrated solutions first obtained separate a solid phase until an equilibrium is apparently established such that less than 1 per cent. of starch remains in a non-opalescent or but slightly opalescent solution (**24, 25**). The phenomenon bears a close resemblance to that exhibited by gelatin and studied by STRAUP (**100**). For different starches, the equilibrium seems to be a little different with respect to the concentration of dissolved starch at the final equilibrium and to depend to some extent upon the total mass of starch in the system. Some starches require a longer time to reach equilibrium than others, and the longer the time the more difficult it is to obtain non-opalescent solutions. These, for example, are more easily obtained from maize and wheat starches than from potato starch (**4, 6, 26**).

Examination with the microscope shows that the dispersal of starch from an injured granule is preceded by localized swelling at the site of the injury; it does not occur anywhere else. The dispersing starch does not stream out

[24] On the other hand, it must be admitted that severe dry grinding affects the properties of gels. Gelatin is but slightly soluble in cold water. If some gelatin such as is used by bacteriologists be ground dry for a long time (50 hours) in a porcelain pebble mill, it yields an opalescent solution when suspended in cold water. This solution filters with difficulty but the small quantity of filtrate is perfectly clear and not very viscous. On standing overnight, it sets to a clear gel (*cf.* 5). It may perhaps also prove significant that SCHOCH (85) prepared dispersions from potato starch ground for 500 hours, which did not readily retrograde, and found that 900 hours of grinding produced a certain amount of dextrinization, as evidenced by reddish iodine coloration. Moreover, TAYLOR and SALZMANN (**104**) found that long grinding in the presence of moisture affected and modified the stability of starch or amylose toward alkali. Possibly such severe treatment is accompanied by a change of some of the starch from the crystalline to the vitreous state as these states are defined by BEILBY (**15**). This change is sometimes accompanied by changes in solubility and in heat of solution. Grinding a crystal or hammering a metal tends to cause some parts of it to flow. If a substance cannot flow by local melting, perhaps it undergoes some localized form of decomposition. Starch does not melt.

into the surrrounding water as though out of a hole in a bag. Rather it behaves as though at the points of injury it were swelling by imbibition. When the imbibition has reached a certain stage, dispersion gradually occurs. The swollen portion never wholly disperses, even after days; nor does the swelling extend into the interior of the granule. The reason why cold-water solubility is related to the degree of disruption of the granules is now clear. Unless the granule has been very completely disrupted, only those portions swell that have been disintegrated; and swelling seems to be a necessary step antecedent to dispersion. It is obvious that the localized dispersion of injured granules is as difficult to explain by the assumption of a membrane or related restraining mechanism as localized swelling—and for the same reasons. It would seem that our hypothesis regarding the structure of granules, if it is satisfactory to explain the swelling phenomena, is equally satisfactory to explain why an intact granule does not disperse, but disperses at once at the site of injury when it is mechanically damaged. Injury having more or less disarranged the close-packed radial parallel chains, some of these are free to associate themselves with water along their long axes and ultimately to disperse. In this connection it may be noted that NÄGELI (**70**, pp. 111–112) reported that saliva attacks granules tangentially, *i.e.*, transversely to the radius of the granule, more readily than in the direction of the radius. This is what one would expect if in fact the starch chains lie radially.

OPTICAL PROPERTIES

Some investigators have assumed that the material in a moist natural granule, being swollen, is under a strain. To this strain, they attribute the appearance of the black cross which is so characteristic when a starch granule is viewed through the polarizing microscope with nicols crossed (**20**). The alleged disappearance of this cross when the granule is dried has been regarded as proof of this view. As HARRISON (**36**) puts it: "Since the granules have a resistant outer coating, any swelling of the interior portions will produce an increase in the internal pressure. When the outer coating is broken, and this occurs when the granules are ground in a mortar, no pressure will be produced by the swelling of the interior portions, but under these conditions no cross is shown by the granules."

HARRISON'S observation that dry starch granules swell when moistened is undoubtedly correct, as we have seen; and so is his observation that disrupted granules may not show the black cross in polarized light. But the assumption which he and many others make that "the granules have a resistant outer coating" has been shown above to be unwarranted. His statement that the black cross becomes indistinct, if the granules are dried, is also unwarranted. PURIEWITSCH (**74**) has reported that arrowroot starch dried for 76 hours at 105° C. has exactly the same appearance in polarized light as wet

starch immersed in water for 2 days. KATZ and DERKSEN (**48**, p. 109) made a similar observation.

It is probable that HARRISON was deceived, because it is difficult to get a clear picture under the polarizing microscope of starch examined in air. Therefore, GRIFFING (**31**), at the writer's suggestion, heated potato starch for several days to a little over 110° C. It was then transferred as rapidly as possible, and still hot, to anhydrous glycerol or to cedar oil and examined immediately in these media; it exhibited perfectly normal birefringence.[25]

It has long been known that when starch is gelatinized by heating in water its birefringence is lost and the black cross disappears. Indeed, the loss of birefringence has been proposed as a criterion for fixing the gelatinization temperature (**76**, p. 298); but WOODRUFF and WEBBER (**110**) found that the temperature at which birefringence disappears varies with the amount of water available for swelling.

As already pointed out, HARRISON (**36**) has shown that the black cross disappears when starch granules swell in the cold as the result of injury. HUSS (**41**) made similar observations and showed that the black cross vanishes whenever the starch granule is made to swell beyond its normal size, whatever the cause. He pointed out that the disappearance of the black cross and the taking up of dyes are parallel phenomena.

Indeed, mechanical disruption greatly modifies the optical properties of granules. HARRISON'S observation that grinding starch in a mortar causes the black cross to disappear has already been mentioned, but the observation is much older (**67**). Indeed, SCHEFFER (**82**) has proposed examination in polarized light as a method of detecting injury to starch granules because such injury is difficult to recognize in ordinary light. In polarized light, injured granules appear either with but slight birefringence or else with only a part of the granule birefringent. The fact that birefringence may be lost only in part of the granule and the black cross remain in part is probably the reason why a few investigators[26] have reported that injury does not obliterate the black cross. The truth seems to be that just as the degree to which a granule is soluble in cold water and swells in it depends upon the completeness of the disruption of the granule, so also does the loss of birefringence. Badly shattered granules lose their black cross and most of their birefringence, but examined with a selenite plate some birefringence may still be detected (**31**), and starch that has been severely ground loses its x-ray diagram (**66, 95**).

The localized loss of birefringence in injured granules may be demonstrated by water extraction of starch ground for many hours in a pebble mill

[25] FISCHER (**27**, p. 83), however, states that dried granules do not show lamination but appear homogeneous, and so does NÄGELI.

[26] For example, NÄGELI (**68**).

until it appears to have lost the black cross. The ground starch is suspended in water and separated from the solution by centrifuging. The process is repeated till the extract is not blued by iodine. The undissolved residue, dried with alcohol and ether and examined in polarized light, appears dull white on the dark field, looking much like the ground starch before it was washed, except that there had been washed away the amorphous, granular, non-luminous material which had been adherent to the surface of the granules. When this material was mounted in cedar oil, the granules appeared dull yellow, transparent, with numerous traces of bright birefringence and distorted black-cross markings. The explanation is obvious. Grinding covered the surface of most granules with abraded starch substance, which is not birefringent or only slightly so, and therefore masked the birefringence of the material in the interior of the granules. After this had been washed away by extraction with water, the granules again appeared birefringent, although this was less pronounced and no longer typical because the granules for the most part were more or less affected by the treatment.

Apparently, anything that sufficiently breaks up the anatomical structure of the granule greatly modifies its birefringence, if it does not wholly destroy it. It is, therefore, probable that at least some of the optical properties are due to parallel arrangement of crystals or other elements in the granule. This is also the opinion of KATZ (**47**).

We must, then, distinguish two sorts of birefringence in the starch granule—that which is due to parallel arrangement of the starch crystals, and that which is inherent in the starch substance itself and still visible with a selenite plate in badly shattered starch. The latter is relatively slight.

The black cross is due to the parallel arrangement of the starch crystallites manifest through the microscope in the radial striations. It is due, therefore, to a comparatively coarse structure and disappears whenever this is disorganized. Hence, it is understandable why the black cross remains in completely anhydrous starch, which, according to KATZ, no longer exhibits an x-ray diagram. In such a dry granule, the radial striation and the laminations are still visible. Conversely, when a starch granule is gelatinized without enough water to permit free swelling,[27] the radial striations, the laminae, and the black cross are no longer visible but the granules still exhibit an x-ray diagram, although an altered one. It is obvious, then, that the disorganization which causes loss of the capacity to exhibit an x-ray diagram is of a finer sort than that which is sufficient to destroy the black cross.

The hypothesis herein advanced is, of course, to a considerable degree a speculation, but a speculation which seems consistent with and capable of offering a reasonable explanation of all the facts known to the writer. It

[27] KATZ terms this "first-degree gelatinization."

emphasizes the organization of the granule without consideration of which it seems impossible to form any sort of rational mental picture of the behavior of the starch granule. The importance of the organization of biological structure in determining behavior has been emphasized by others (**40**). The hypothesis offered may in the end prove to be ill-founded, but it poses many questions which can be answered by experiment and this is sufficient justification for its presentation.

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A CHEMICAL AND PHYSIOLOGICAL STUDY OF TRAUMATIN, A PLANT WOUND HORMONE

JAMES BONNER AND JAMES ENGLISH, JR.

(WITH THREE FIGURES)

Introduction

The first tentative formulation of the "wound substance" concept was made by WIESNER some 50 years ago. In his "*Elementarstruktur*" (**25**) he suggested that it might well be that *substances* formed or produced by wounded cells pass from these cells to neighboring uninjured tissue and there bring about such phenomena as callus formation and regeneration; bring about, in short, a resumption of meristematic activity by cells apparently mature. Sound experimental evidence in support of the wound substance hypothesis was obtained by HABERLANDT (**5–9**). His classical potato experiment should be particularly stressed. He found that discs cut from a potato tuber showed cell divisions leading to periderm formation only if (a) phloem and (b) "wound hormone" were present. This wound hormone appeared to come from contents of injured cells at the cut surface of the disc. In a number of different ways HABERLANDT demonstrated that both the influence coming from the phloem and that coming from the surface are diffusible chemical substances and that the interaction of the two is necessary for renewed division of the mature parenchymatous cells of the potato tuber, as well as of the kohlrabi root (**8**). In a later publication (**8**) HABERLANDT showed that in other cases also, division of mature cells may be induced by the cooperation of "lepto-hormone" and "wound hormone." By judicious dissection of the leaves of succulents, surfaces of uninjured cells could be exposed. The cells of these uninjured surfaces were capable of responding to the application of tissue juice from other leaves with a vigorous renewal of cell division activity.

REICHE (**19**) confirmed the results of HABERLANDT in another way. She injected the petioles and stems of various plants (Nymphaeaceae, *Solanum*, *Gratiola*) with dilute tissue extracts and found that cell divisions were induced wherever the *uninjured* cells of the stem or petiole came in contact with the extract of *injured* cells. She was inclined to the view, however, that the activity of the extracts resided in suspended cell fragments rather than in soluble substances. WEHNELT (**23**), whose work with the parenchymatous lining of bean pods will be discussed more extensively later, found that these intact parenchymatous cells react rather to a water soluble, heat stable substance present in tissue extract. Another favorable object for the demonstration of wound hormone activity was found by WILHELM (**26**) in the parencyhmatous lining of the hollow stem of *Vicia faba*.

It may be concluded that it is a well established fact that when a plant is injured, substances are formed or liberated which are capable of causing other uninjured cells to resume active growth. These substances may well be called wound hormones since they are carriers of correlation between one portion of the plant and other portions. They appear in some cases to be also "cell division substances." It is equally well established that in many cases the wound hormone acts only in conjunction with a second factor contained in the phloem. This factor, however, is less well known and is less amenable to study than is the wound hormone itself.

The chemical nature of the wound hormone has not been studied previously in any detail. In fact, its very existence as a chemical individual might be considered as thrown into doubt by the work of WEHNELT and WILHELM, who have shown that the most diverse and ill assorted substances possess some typical wound hormone activity. The chemical properties of the active principle of tissue extract are also in question since it has been reported by HABERLANDT that it is heat labile, by WEHNELT that it is heat stable, and by REICHE that it is not soluble in water but is heat stable. In this connection it should be remembered that different test objects may actually respond to different wound hormones.

That the wound hormone possesses considerable interest both from the theoretical and the practical points of view is perhaps obvious. In the wound hormone we have a substance which is capable, under suitable circumstances, of bringing about renewed growth activity of otherwise mature cells. The rôle of wound hormone in wound healing, parthenogenesis, adventive embryony, callus formation, etc., has been discussed in detail by HABERLANDT (**9**) and need not be gone into here. Attention has been called more recently to the rôle of wound hormone in the culture *in vitro* of plant tissues (**2**). In the present work, an attempt has been made to work out *one quantitative* test for wound substance activity, and, using this test, to purify the active principle. The work, therefore, has been confined to one test object. The test was developed from that suggested by WEHNELT (**23**). Since the work was undertaken in the fall of 1935, several papers bearing on the subject have appeared (**13, 15, 17, 20, 22**). These will be discussed later as the appropriate connections arise.

Experimentation

PRINCIPLE OF THE TEST

The use of the immature bean pod for the demonstration of the action of wound hormone is due to WEHNELT (**23**), who worked out a qualitative activity test and determined a number of facts concerning the physiology and chemical properties of the active principle.

The immature bean pod merely need be slit lengthwise along suture and

midrib, and the unripe seeds removed, to expose the layer of uninjured parenchymatous tissue which lines each seed chamber. If this layer of tissue is then injured, for example, by a prick with a needle, the cells in the region of the wound divide and enlarge so that a small "neoplasm" or intumescence projecting above the level of the surrounding tissue is formed. A much more striking result may be obtained by the application of juice from crushed beans. If a drop of such bean juice is applied to the uninjured surface it is absorbed in about 24 hours. Under the point of application of the drop and before it is completely absorbed, a cylindrical intumescence begins to arise, which may attain a height of as much as 3 mm. in the course of 48 hours. Histologically the intumescence consists of cylindrical parenchymatous cells elongated at right angles to the surface of the seed chamber.

The change in wall structure of these cells during the course of the reaction is a point of some interest. The normal parenchyma of the seed chamber consists of approximately isodiametric cells and these possess the foliar arrangement of cell wall micellar units which is typical of such cells (**1**). They are isotropic when viewed in tangential section between crossed nicols of the polarizing microscope. During the course of the wound hormone reaction the shape of these cells changes to cylindrical due to the extensive cell elongation which takes place. The elongating cells are anisodiametric and they show the corresponding anisotropy of cell wall structure which is typical of elongating cells. The micellar units are arranged in a direction which is, statistically, perpendicular to the axis of elongation (**1**).

Normal mitotic cell divisions occur in the new growth, their frequency depending upon the species and variety of bean used. In a given variety of bean the frequency of cell division depends upon the concentration of the applied extract as does the height of the intumescence. With increasing size, the total number of cell divisions increases. The intumescence is thus a *product* of simultaneous cell division and cell enlargement. In our experience those varieties of beans which give large intumescences in response to the addition of wound hormone also show extensive cell division, whereas those varieties which give intumescences of limited size may show few or no cell divisions. As far as has been observed during the present work, cell divisions appear to be essential to the formation of large amounts of new growth. Previous authors who have investigated the wound hormone response of the bean pod have interested themselves chiefly in this cell division activity (**23, 13**). The quantitative estimation of the latter, however, is at best tedious and hence difficult of application to large scale routine testing. Furthermore, investigators who have *confined* themselves to estimation of division frequency, as will be shown later, have been misled on important points. In the present investigation the *height* (size) of the intumescence, a readily and quantitatively determinable quantity, has been chosen as the criterion of wound substance activity.

THE QUANTITATIVE TEST

It was found quite possible, with the observance of a number of precautions, to place the "bean test" upon a quantitative basis. A large and constant supply of beans was essential. This was obtained through the cooperation of the Lake Farm Produce Co.[1] The beans (Kentucky Wonder) to be used on any given day were selected from a fresh, high grade stock (one pound chosen from 50 to 150 pounds). Only uniformly firm, immature (not rounded out by the developing seeds) pods of dark-green color were selected. On those days when fresh beans were not available, stock which had been stored at 35° F. was used. Such pods, however, gave less reliable results, apparently owing to the fact that they were extensively dried out. Tests were made at the same hour each day in order to avoid, among other things, effects of diurnal fluctuation such as are found in the *Avena* test (**14**).

The pods were slit lengthwise along suture and midrib (fig. 1), opened, and the seeds removed. The individual seed chambers were then cut from the pod and arranged in Petri dishes upon moistened absorbent paper. The apical chamber and the basal one from each pod were rejected since these are less reactive than the others. The "cups" were arranged in five columns of six cups each. Each column consisted of chambers from a single bean; each row, of chambers from different beans. In each Petri dish there were, therefore, cups from five different beans, six cups from each bean, and in each dish it was possible to test six different solutions on five different beans. Because five beans did not suffice in general to give an accurate measure of activity, due to individual variations, ten beans were used in most determinations. In tests requiring special accuracy (as, for example, molecular weight determinations by the diffusion method (**4, 24, 12**) twenty or more beans were used. The solutions to be tested were diluted with distilled water instead of nutrient solution, since it was found that the use of a balanced salt solution made no difference in the results. Drops of the test solution (approx. 0.01 cc. per drop) were placed in the center of each cup with a micropipette. The tests were then placed in an incubator at 25° C. At the end of the test period (in general 48 hours, see below), each cup was sectioned through the reacting portion and the height of intumescence measured under a low power binocular microscope.

COURSE OF THE REACTION WITH TIME

Figure 2 gives the course of the reaction with time for a series of dilutions of the same stock solution. The first reaction appears between 8 and 16 hours, before the drops have been completely absorbed. Each concentration

[1] The authors are deeply indebted to Mr. K. WADA of the Lake Farm Produce Co. Only through his constant cooperation and assistance was it possible to obtain the supply of fresh high quality beans needed for the routine testing.

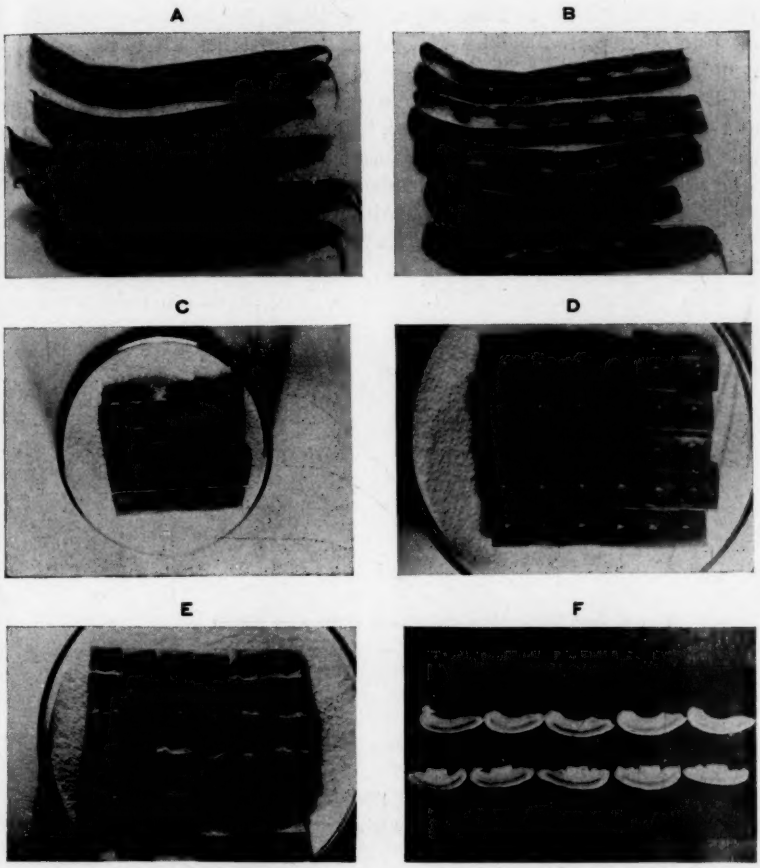

FIG. 1. Steps in the execution of the "bean test": A, fresh beans; B, beans opened and seeds removed; C, individual seed chambers arranged in Petri dish; D, drops (containing traumatin) in place; E, characteristic reaction to traumatin, after 48 hours; F, cross section through the seed chamber after 48 hour test. The top row is a typical control, and the bottom row a reaction which is shown in the linear portion of fig. 3.

gives a typical S-shaped growth curve, the curves from tests with smaller concentrations having lesser slopes and earlier maxima than those from tests with the higher concentrations. The highest concentration, for example, attained its maximum only after 64 hours while the lowest concentration attained its maximum after 40 hours. Since, for routine testing, it was desirable to have as short a test period as possible, 48 hours was chosen as

the standard length of incubation. Figure 3 gives a typical example of concentration plotted against height of intumescence after 48 hours. It may be seen that:

1. Height is proportional to concentration between approximately 0.20 and 0.67 mm. Over this range, then, height of intumescence, less the correction due to the fact that the curve does not pass through the origin, may be used as a measure of wound hormone activity. If a longer time is chosen, the linear relationship holds over a wider range.

2. The curve does not pass through the origin. If the main linear portion is extrapolated, it passes through the height axis at about 0.18 mm.

This "intersection point" (I, on fig. 3) is of fundamental importance to

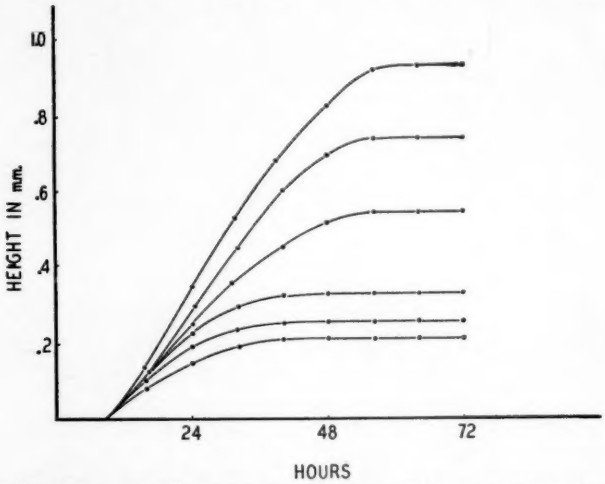

FIG. 2. Development of intumescence with time. Six concentrations of traumatin, each one-half as great as the preceding, are represented.

the understanding of the test. The height of intumescence corresponding to the intersection point varies from day to day, but on any given day it is equal to the maximum response which is given to any of the "non-specific" agents which are discussed below. It is to be noted, however, that non-specific agents may cause a response *smaller* than this; thus water causes a response which is several times smaller than the *maximum* non-specific response which is possible. Above this non-specific portion of the response is superimposed the major effect of the added hormone. Only those substances which in some concentration elicit a response greater than the non-specific portion, can be considered as possessing true wound hormone activity. The failure in the past to distinguish sharply between specific and non-spe-

cific response may have been due to the much smaller accuracy of the cell division frequency determinations which have been made. In any case it has led to the present confusion as to the specificity of the hormone, a question which will be discussed later.

The wound hormone itself is capable of causing the non-specific as well as the specific response. That this is so, is clear from the activity-concentration curve (fig. 3) in which one is superimposed on the other. The non-specific

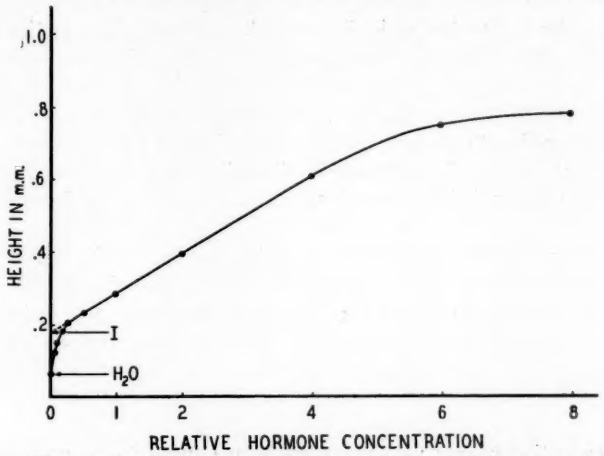

FIG. 3. The relation between traumatin concentration and height of intumescence. I, "intersection point" (see text); H_2O, the reaction to pure water.

portion is brought about by much lower concentrations of wound hormone than is the specific response, and also it is caused by much smaller concentrations of wound hormone than of the various non-specific agents. This fact suggests a second measure for wound hormone activity, namely, the lowest concentration which is just capable of causing full non-specific response. This measure of activity does *not* give relative activities for two wound hormone preparations identical with those obtained by the first method (from large intumescences), as will be shown later.

THE NON-SPECIFIC REACTION

It has already been mentioned, that WEHNELT (**23**) found a number of agents other than the juice of beans, capable of causing a reaction in the bean test. WEHNELT was often troubled with a considerable reaction caused by the application of drops of plain water. He also found that sugars, neutral salts, and even agar regularly caused reaction. It is desirable to make clear at once the relationship between these non-specific reactions

and those due to the application of wound hormone. Water alone gives a reaction particularly if the test tissue is relatively dry. The standard "fresh" beans[2] which have been used in this investigation give, in general, either no response or a negligible one to the application of pure water (0.07 mm. is a value obtained on many days). If such beans are allowed to dry out, however, they react more vigorously to water. Sugars and neutral salts give, with these beans, reactions greater than that of water only when used in high concentrations. Thus 10 per cent. glucose gave a reaction four times as great as that of water, while 1 per cent. gave only the reaction of water alone. The more permeable substance, urea, in equivalent concentrations gave reactions only half as great as those due to glucose. The effects of these substances are clearly osmotic ones. Another class of non-specific responses includes those due to wounds or to the application of markedly toxic substances. In either of these cases there is a circular zone of response around the dead or badly injured portion, and a crater-shaped intumescence is formed.

It is clear that the non-specific causative agents may be classified under two general headings: (1) Those causing slight if any visible injury such as hypo- and hyper-tonic solutions; (2) those causing severe injury, either mechanical (pricks, etc.) or chemical ($HgCl_2$, strong acid or alkali, etc.). In these cases it is clear from the crater shape of the intumescence that the injured cells have liberated wound hormone which caused adjacent cells to react.

The causative agents which superficially appear to be non-specific are probably all based upon the liberation of wound hormone within the tissue itself. The maximum amount thus liberated by agents which are not visibly toxic is then just sufficient to cause a maximum intersection point non-specific response. Intumescences caused in this way are not to be confused with those caused by the addition of wound hormone from outside. The average maximum response due to the former is 0.10 to 0.30 mm., that due to the addition of wound hormone, 1.9 mm. or more.

Reactivity of different varieties of beans

A number of leguminous pods other than those of *Phaseolus* were tested as to their suitability for the bean test. Pods such as those of pea, lima bean, and chinese pea, which have only rudimentary parenchymatous linings of the seed chambers give no reaction whatsoever. This is not surprising since the reaction is carried out by the parenchymatous lining of the bean. Different varieties of the string bean itself also differ in their response to wound hormone. The maximum average height of intumescence for five varieties

[2] By this is meant fresh market beans. Beans fresh from the plant were found to be improved by a slight amount of drying (2).

is recorded in table I. Kentucky Wonder, brown seed, is at least four times more reactive than is Kentucky Wonder, white seed, and excels the other varieties still more. The brown seed Kentucky Wonder was, therefore, used exclusively for the quantitative test outlined above.

TABLE I

MAXIMUM HEIGHT OF INTUMESCENCE GIVEN BY DIFFERENT VARIETIES OF BEANS

VARIETY	MAXIMUM AV. INTUMESCENCE HEIGHT
	mm.
Kentucky Wonder, brown seed	1.9
Kentucky Wonder, white seed	0.45
Florida Black Valentine	0.3
Golden Wax	0.15
Green Pod	0.00

Some investigation of the reasons underlying this varietal difference in reactivity has been made. In table II are given the results of reciprocal tests between Kentucky Wonder brown seed, and Florida Black Valentine. The

TABLE II

RECIPROCAL TESTS BETWEEN KENTUCKY WONDER AND FLORIDA BLACK VALENTINE BEANS

EXTRACT USED	HEIGHT OF INTUMESCENCE	
	KENTUCKY WONDER BEANS	BLACK VALENTINE BEANS
	mm.	*mm.*
Kentucky Wonder	0.65	0.30
Black Valentine	0.51	0.30
Kentucky Wonder + Black Valentine	0.66	0.30
Free Acid Conc.	0.70	0.0

latter contains wound hormone which is highly active on Kentucky Wonder but only slightly active on itself. Kentucky Wonder juice also has only slight activity on Valentine. A mixture of the two juices shows that Valentine juice contains no inhibitor of the reaction. The fraction of the juice which is active on Kentucky Wonder and which was purified during the course of the present work possesses little, if any, activity on Valentine. A possible explanation is that the interaction of several factors is necessary for the response and that we have purified only one of them. The others, apparently, are present in the Kentucky Wonder bean in considerable amount, but are lacking in the Valentine. A complete elucidation of the reasons for the difference must however await a further investigation.

Distribution of the wound hormone

That the wound hormone is widely found in nature has been indicated by the work of Wehnelt (**23**) as well as that of others. A more extensive investigation of its distribution has been made during the course of the present investigation and a portion of the results are presented in table III.

TABLE III

Occurrence of the wound hormone

Source	Activity	Source	Activity
Bean pod	++	Corn meal	−
Brussels sprouts	++	Soy bean meal	−
Sweet potato	+	Wheat germ	+
Potato	+	Molasses	+
Orange	++	Yeast, bakers'	−
Lemon	++	Yeast, brewers'	+
Tomato	++	Vitamin B concentrate	−
Lettuce	++	Urine, human	−
Spinach	+	Urine, cow	−
Pea plant, etiolated	++	Peptone, Difco	−
Pea plant, green	++	Beef extract	−
Pea seed	−	Liver extract	−
Hay, alfalfa	+	Milk	−
Malt	+	Egg albumin	−
Rice polishings	−	Serum	−
Cabbage	+	Emulsin	−

Each source was tested over a wide range of concentrations. A substance listed as inactive failed to cause any response other than non-specific in any concentration. Of the animal tissue and extracts examined, none was found to possess any marked activity. The reaction to serum obtained by Wehnelt was, presumably, merely non-specific in nature. The urine of cows is of particular interest. Alfalfa hay is a relatively rich source of the hormone (table III). The urine from cows fed this hay, however, is completely inactive. Wound hormone behaves, in this respect, quite differently from auxin a, which is ingested by the animal and excreted in the urine. The "sporogenes vitamin" (**18**) also is found in the urine of herbivores. Wound hormone, however, either is destroyed by the flora of the gut, or is metabolized further by the cow.

The richest sources were found to be green beans themselves, leaves in general, dry brewers' yeast, and the juice of oranges and tomatoes. Attention might be called to the fact that various samples of brewers' yeast were all inactive, in marked contrast to dry brewers' yeast. Although green portions of the plant are rich in wound hormone, the latter is not confined to

chlorophyll-containing parts. Thus, it is to be found in fruits, in potatoes, and in wheat germ. It is not present in any considerable amount in the pea seed, but is formed after germination and during growth of the seedling. Seedlings grown in darkness contained as much wound hormone per unit dry weight as did those grown in the light.

Table IV gives a quantitative comparison of a few of the more promising

TABLE IV

A QUANTITATIVE COMPARISON OF SOURCES RICH IN WOUND HORMONE

METHOD OF EXTRACTION	SOURCE	RELATIVE ACTIVITY PER MG. DRY WT. OF EXTRACT
Water extraction	Bean pod	100
	Pea plant	77
	Orange	50
	Yeast, brewers'	31
	Alfalfa hay	15
Absolute alcohol extraction	Bean pod	410
	Yeast, brewers'	380
	Alfalfa hay	233
	Wheat germ	143

sources of the hormone. In no case was a source found which was richer than the bean pod itself. This is in accord with the findings of SILBERSCHMIDT and KRAMER (**20**), that the wound hormone content of a plant tissue, as measured by the bean test, is greater the closer the relationship of the plant in question to the bean. However, brewers' yeast which is certainly systematically far removed from the bean yields a very active alcoholic extract.

It might be mentioned here that a number of different methods of extraction of the wound hormone have been compared. The results of a typical experiment are presented in table V. The hormone is heat stable and the

TABLE V

A COMPARISON OF DIFFERENT METHODS OF EXTRACTING WOUND HORMONE FROM BEAN PODS

METHOD OF EXTRACTION	PREPARATION OF MATERIAL	RELATIVE ACTIVITY	YIELD: % DRY MATTER/DRY WT.	TOTAL AMOUNT OF HORMONE
Water extraction	Fresh: ground	1	41	41
	Ground: dried	1	44	44
	Dried: ground	1	48	48
Alcohol extraction	Ground: dried	4.1	4	16.4
	Dried: ground	4.1		

fresh beans may be dried and extracted without loss of activity. It is of no advantage to grind the tissue (thus to wound it severely by mechanical means) before drying and extracting. Of the organic solvents used for this initial extraction, absolute ethyl alcohol was found to be the most effective. The extract which is obtained by extraction with hot alcohol is more *concentrated* in wound hormone than is the corresponding water extract, although a smaller *total amount* of hormone is obtained, even if the alcohol extraction is apparently complete. The alcohol extraction possesses other advantages (**4**), and has been used in the subsequent procedure for purification.

Specificity of the reaction

A number of pure substances, known to possess physiological activity upon plants, were tested during the course of the work. A partial list of these and other pure substances tried is given in table VI. None of the com-

TABLE VI

Pure substances found to be inactive in the bean test

Indole-3-acetic acid	Cystine
Aneurin, (Vitamin B_1)	Cystine
Lacto-flavin	Alanine
Ascorbic acid	Glycine
1–2–5–6–di-benzanthracene	Methionine
Folliculin	Tyrosin
Pantothenic acid*	Histidine
i-Inositol	Proline
Biotin†	Valine
	Tryptophane
	Leucine
Lecithin	Asparagin
Tannic acid	Aspartic acid
Hesperidin	Glutamic acid
Narinigen	Ornithine
Colchicine	Lysine
Allantoin	Serine
	Betaine

* Obtained through the generosity of Prof. R. J. Williams, Corvallis, Oregon.

† Tested on different but reactive beans by Prof. F. W. Went. Biotin supplied through the courtesy of Prof. F. Kögl, Utrecht.

pounds elicited any response other than the non-specific or that due to marked toxicity in the higher concentrations. Of particular interest are the following:

Hetero-auxin, mentioned by Jost (**13**) as possessing activity in the bean test. Jost, however, used very high concentrations (1:1000) which apparently were toxic. Hetero-auxin was inactive in the present test.

Aneurin (vitamin B_1), a growth factor for other plant tissues (3) but inactive in the bean test.

Biotin, pantothenic acid, and *i-inositol,* substances of the yeast "bios" group. LAIRD and WEST (15) have found a "bios 2b concentrate" to be active upon their beans. Pure or nearly pure "bios 2b" (biotin, pantothenic acid), however, were inactive in the present test. Prof. R. J. WILLIAMS, furthermore, has very kindly tested our fraction VII-b (below) under a number of conditions and found it to have only negligible "bios" activity.

Tyrosin, said by ORSOS (17) to be the active wound hormone principle in the kohlrabi test of HABERLANDT. It was inactive in the present test, as were all of the amino acids tested, both alone and in combination.

Ascorbic acid, which, according to HAVAS (10) plays some rôle in abnormal plant growth. It was inactive in the bean test.

Colchicine, said by HAVAS (11) to promote abnormal meristematic activity. It was inactive in the bean test.

PURIFICATION OF THE WOUND HORMONE

The quantitative test was developed with the particular view of using it as a tool in the isolation and chemical identification of the wound hormone. Each step in the purification was arrived at only after much preliminary groping with this physiological assay as a guide. The evidence for the value of each step cannot be given here. Since a detailed account of the procedure and a discussion of the chemical properties of the products may be found elsewhere (4), only a brief résumé is presented in this paper.

The activities of the various fractions are given below in two ways: (a) that determined from the concentration needed to give a large intumescence (0.30 to 0.70 on the scale of fig. 3), given as activity per mg. relative to the activity per mg. of the initial alcohol extract as unity; and (b) that determined from the minimum concentration giving an intersection point response. This is called the minimum active concentration.

Three separate lots of beans, 100 pounds in all, were extracted. The summary given in table VII is for the extraction of one of these lots, having an initial wet weight of 20 pounds.

It was impossible to increase significantly the activity of VII–b by further extractions or precipitations or even by preparation and distillation of the methyl ester. This free acid concentrate of VII–b is a yellow, extremely hygroscopic, amorphous solid, which is insoluble in ether, chloroform, etc., but readily soluble in water, alcohol, pyridine, etc. It is heat, acid and alkali stable, and contains basic nitrogen although not as a primary amine. The molecular weight as determined by titration, diffusion (4, 12), and by other methods, is in the neighborhood of 220. The ester product of this "free acid concentrate" is a yellow oil which dis-

TABLE VII

RÉSUMÉ OF STEPS IN THE PURIFICATION OF THE ACTIVE PRINCIPLE OF THE WOUND HORMONE

PROCEDURE	AMOUNT	ACTIVITY PER MG. RELATIVE TO I–b	MINIMUM ACTIVE CONCENTRATION
I. Extraction of dried beans with absolute alcohol			
a. hot water extract of residue	.....	0.0	.....
b. water soluble of alcohol extract	92 gm.	1.0	1: 400
II. Adsorption of I–b with charcoal and elution with pyridine			
a. charcoal filtrate	42 gm.	0.0	.....
b. combined eluates	52 gm.	1.5	1: 1600
III. Fractional ether precipitation of II–b from pyridine			
a. ether precipitate	22 gm.	0.0	.....
b. ether filtrate	30 gm.	1.4	1: 4500
IV. Extraction of III–b with ethyl acetate			
a. ethyl acetate insoluble	17 gm.	0.3	.....
b. ethyl acetate soluble	12 gm.	2.0	1: 12800
V. Conversion of IV–b to its barium salts, alcohol extraction, and regeneration of the free acids			
a. alcohol soluble	3.8 gm.	1.6	.....
b. alcohol insoluble	6.4 gm.	6.3	1: 36000
VI. Precipitation of V–b with mercuric acetate and subsequent regeneration with H_2S			
a. mercuric acetate filtrate	3.0 gm.	2.8	.....
b. mercuric acetate precipitate	1.62 gm.	10.8	1: 100,000
VII. Extraction of VI–b with acetone			
a. acetone insoluble	0.216 gm.	10.3	.....
b. acetone soluble	1.406 gm.	13.8	1: 100,000
VIII. Preparation and high vacuum distillation of the methyl ester of VII–b			
a. unchanged free acid	25% of VII–b	13.1	.....
b. main fraction of ester distillate (after hydrolysis)	15% of VII–b	14.4	1: 100,000

tilled well and could be redistilled repeatedly without change of activity. It should be noted that the ester can be tested only after hydrolysis, since it is water insoluble. The products of the three separate extractions gave identical analytical results, the composition of the redistilled ester agreeing approximately with the formula $C_{11}H_{17}O_4N$. It seems probable that this product is not far from pure although it has not as yet yielded a single crystalline derivative.

From the 100 pounds of fresh bean which were extracted a total of approximately 500 mg. of distilled ester were obtained. This amount has been too small to permit of further investigations, but it is planned to carry out the extraction upon a larger scale in the near future.

Discussion

The procedure used for the purification resulted in an enrichment of the active principle of about 250 times over that of the initial alcohol extract, as measured by the minimum active concentration. As measured by the concentration required to produce a given large size of intumescence, the enrichment, however, was only 14 times. Although no portions containing any significant amount of activity were discarded during the procedure, nevertheless the enrichment as measured in this way was much less than the enrichment of material, which was 65 times between alcohol extract and product VII–b. At least two possible explanations suggest themselves:

1. The wound hormone may be continuously altered to a closely related inactive substance.

2. The cooperation of a second substance or substances may be necessary for the production of large intumescences. Such substances should need to be inactive alone, but should augment the effect of the fraction which we have purified.

With regard to the first possibility, it has been impossible to demonstrate that spontaneous inactivation of the wound hormone occurs, although this has been looked for repeatedly. The second possibility also has been borne in mind constantly. At each step of the procedure the two resulting fractions were tested not only separately but *in combination*. In no case was it possible to find any synergistic action of the combined fractions. The free acid concentrate was also tested in combination with other fractions or substances, for example, with charcoal filtrate of fresh extract, fraction II–a, sugar, aneurin, lactoflavin, and hetero-auxin. The slightest evidence for any co-wound hormone activity was never detected.

UMRATH and SOLTYS (**22**) in a paper which has appeared since the present work was undertaken, have also used the bean test (although in a less quantitative fashion) as an aid for the enrichment of wound hormone. They obtained, from alfalfa, a product having an activity of 1:50,000, or one only slightly less active than that obtained here. Chemically, however, the products differ, that of UMRATH and SOLTYS containing less nitrogen and much more oxygen than that reported here, and apparently being more closely related to the "Mimosa substance" of SOLTYS and UMRATH (**21**). It seems possible that the apparent difference in composition of the two substances may be due only to a difference in their respective purities.

As we have mentioned in the introduction, HABERLANDT (**8**) found that

a substance coming from the phloem (lepto-hormone) interacts with the wound hormone in the induction of cell divisions, at least in certain cases such as in the potato tuber. In the case of the bean test, it may be supposed that the lepto-hormone, if it is necessary, may be supplied by the vascular tissue of the pod. Over the range in which response is proportional to applied wound hormone concentration, it is in any case clear that wound hormone rather than lepto-hormone is the limiting factor in the normal bean test. Tests were therefore carried out in which it was attempted to make lepto-hormone the limiting factor. The seed chamber was completely freed of vascular tissue and tests with high concentrations of wound hormone were carried out on the isolated parenchyma. Removal of the vascular tissue actually did reduce the response greatly. If the isolated parenchyma was placed upon agar containing sugar, it responded much more vigorously, although still not as well as the intact controls. One might be tempted to conclude that lepto-hormone, in the case of the bean, consists principally of nutrient sugars. However, the situation is actually more complicated and can be worked out fully only when larger supplies of pure wound hormone are available.

With the isolation of an active wound hormone, even though it may not as yet be in a completely pure state, it is proper to propose a chemical name for the substance. The entire background of the subject from HABERLANDT, in particular, to the present, would seem to make "traumatin" particularly appropriate. It should be stressed again that there may be many wound hormones, that different test objects respond to different chemical substances, and that the present investigation has been concerned with traumatin, *a* plant wound hormone, rather than with traumatin, *the* plant wound hormone.

Summary

1. The origin and development of the wound hormone concept have been reviewed briefly. Of the several available methods for the demonstration of wound hormone activity, that of WEHNELT (**23**) was chosen for the present work.

2. A quantitative assay of wound hormone activity has been described. In this test, *size* of the new growth rather than frequency of cell division is measured.

3. The test was shown to be specific for this wound hormone.

4. With the aid of the quantitative assay it was possible to isolate a substance, apparently not far from pure, and possessing typical wound hormone activity. For this substance the name traumatin is proposed.

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EFFECT OF CERTAIN IONS, USED SINGLY AND IN COMBINATION, ON THE GROWTH AND POTASSIUM, CALCIUM, AND MAGNESIUM ABSORPTION OF THE BEAN PLANT

ROBERT L. CAROLUS
(WITH TWO FIGURES)

Introduction

Almost every season a considerable portion of the acreage of edible podded beans in the Norfolk section, although adequately fertilized with nitrogen, phosphorus, and potash, fails to produce a satisfactory crop because of the fact that some nutritional phenomenon interferes with normal plant growth. As the trouble has occurred in many cases only in certain sections of a field, the poor growth generally could not be attributed to unfavorable climatic conditions. A recent study of magnesium deficiency (**3**), in which chemical analyses of the plants indicated that in some cases ions other than magnesium altered the absorption and utilization of the magnesium ion by the plant, suggested that some similar phenomenon causing an unbalanced condition in the soil or in the plant might be responsible for the observed unsatisfactory development of the bean crop.

The study of various ions and their interrelations, both in the plant and in the soil, has produced voluminous experimental evidence regarding the rôle of various ions in plant nutrition and plant growth. However, as most of these studies have been conducted with water or sand cultures, the concomitant effects of the soil on these phenomena have not been clearly identified. In the work reported in this paper an attempt will be made to indicate some of the factors, both in the plant and in the soil, that may be partly responsible for the differences in plant response observed in beans grown under conditions produced by the addition of various ions, both singly and in combinations, to the soil. By supplementing the regular tissue analysis of the entire plant with an analysis of the metabolically more active sap of the stems of the plant, a new criterion for determining the effects of the various ionic combinations on both current absorption and total utilization was set up, which may possibly be of material aid in clarifying some of the physiological and chemical relationships existing between the various ions.

Experimental methods

Seeds of the Bountiful variety of the edible podded string bean (*Phaseolus vulgaris*) were planted September 9 in two-gallon glazed, drained pots filled with a medium composed of one-half soil, classified as a Norfolk fine sandy loam, and one-half washed sand. In colloid and organic matter content this medium probably is comparable to the poorest type of soil in this

section. A test of this medium by the Soils Department, using methods fully described elsewhere (**6**), indicated a soil reaction of pH 5.1, a good rating for available phosphorus, potassium, and ammonia nitrogen, and a low to fair rating for magnesium. The amounts of the various cations (expressed in terms of their oxides) found in the replaceable state at the end of the third crop, and after a heavy leaching in a pot containing 9 kg. of the unfertilized soil, were 15.9 gm. of potash, 2.8 gm. of calcium oxide, and 1.25 gm. of magnesium oxide.

Each fertilizer treatment, as listed in table I, was applied, after the plants had emerged on September 22, to duplicate pots at a rate equivalent to 500 pounds per acre of a fertilizer analyzing 6 per cent. nitrogen, 6 per cent. phosphoric acid, and 5 per cent. potash. All treatments except number one contained equal amounts of nitrogen and all except numbers one, two, and four contained equal amounts of phosphorus. All cations were applied in equivalent weights. Consequently, those containing two, three, or four cations contained two, three, or four times as much equivalent cation material as those receiving a single cation.

The plants were harvested October 15 when they had reached the blooming stage shown in figures 1 and 2. Determinations of K_2O, CaO, and MgO in the fresh plant tissue were made as follows: Five-gram portions of finely cut stem tissue taken from plants given each of the treatments were ground in mortars with 20 cc. of 2 per cent. acetic acid and approximately 0.25 gm. of charcoal for 10 minutes, and then filtered at once. Portions of this extract were used in testing for potash with the aid of sodium cobaltinitrite, for calcium oxide with the aid of sodium oxalate, and for magnesia with a weak alcoholic solution of titan yellow. The turbidity developed in the potash and lime test, and the progressive changes in color for different concentrations of magnesia were made the basis of comparison with solutions containing known amounts of potash, lime, or magnesia. Numerous details involved and precautions used in making these tests have already been published (**2, 5**) and will not be reiterated here.

The roots, leaves, and remainder of the stems from each treatment were dried and subjected to the conventional tissue analyses for magnesia, calcium oxide, and potash (**1**).

Experimental results

Growth response

In figures 1 and 2, examples of the type and extent of the growth produced under most of the ionic treatments used are shown at the period just prior to sampling for chemical analysis. In the upper row of crocks in figure 1, the general debility and lack of plant growth are evidences that some factor or factors interfered with normal development. Similar conditions of

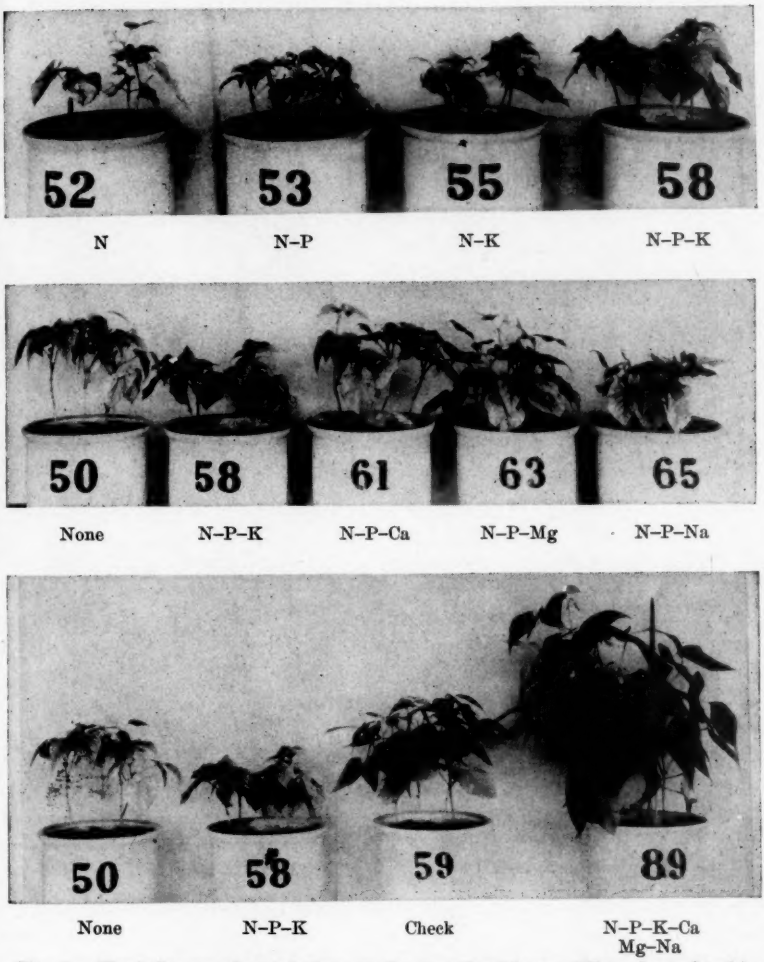
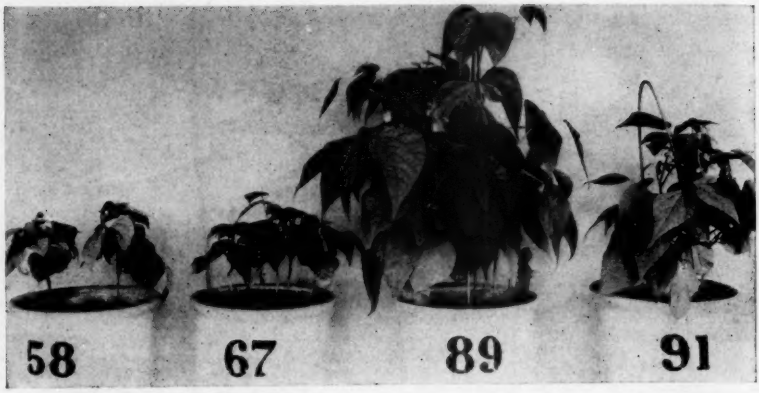


FIG. 1. The influence of certain ions on the growth of beans (*Phaseolus vulgaris*).

chlorosis, dwarfing, and early leaf shedding are often observed under field conditions, and probably are caused by some unbalanced nutrient condition in the soil. In the center row of pots in figure 1, the unfertilized plants (pot 50) are taller than many of those grown in treated pots. However, their dry weight was not equal to that produced by any of the others shown in the center row, but was greater than that produced in pots receiving either N, N–P, or N–K mixtures. The dwarfing of the stems and petioles in the bean



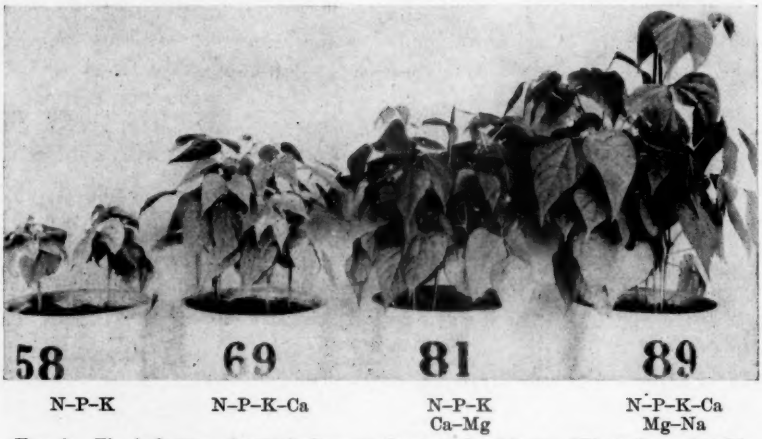


FIG. 2. The influence of certain ions on the growth of beans (*Phaseolus vulgaris*).

plants that received only the sodium cation was a very characteristic symptom of sodium toxicity and has been observed in many other crops grown under similar conditions. In the lower row the effect of a regular commercial fertilizer (pot 50) as contrasted with one that contains only N, P, K, Ca, Mg, and Na ions (pot 89) is shown. For the best growth of beans some of the ions usually included in a regular commercial mixture evidently should be omitted. As the commercial mixture contained considerable sodium from nitrate of soda, an abundance of sulphate from sulphate of ammonia, and all the potash from potassium chloride, the sodium, chlorine, and sulphate ions

probably were partially responsible for some of the differences in growth that were observed. In the upper series in figure 2 this idea is substantiated for the chlorine and sulphate ions. Pot 91 received the same cations as were used in pot 89, but, in addition, received some chlorine and sulphate ions which probably were responsible directly or indirectly for the observed differences in growth.

In the lower series of pots in figure 2 the detrimental effect of cation unbalance is readily observed. Growth was more responsive to fertilizer containing all four cations than to any mixture from which one was omitted. Although beans respond positively to a mixture of all four cations, the potato,

TABLE I

CHEMICAL COMPOSITION OF FERTILIZERS USED

TREATMENT NO.	DESCRIPTION	CONSTITUENTS AND AMOUNTS APPLIED TO EACH POT (U. S. P. CHEMICALS USED)
1	None	None
2	N	NH_4NO_3, 1.39 gm.
3	N–P	NH_4NO_3, 1.12 gm. + $NH_4H_2PO_4$, 0.79 gm.
4	N–K	NH_4NO_3, 1.04 gm. + KNO_3, 0.88 gm.
5	N–P–K	NH_4NO_3, 0.77 gm. + $NH_4H_2PO_4$, 0.79 gm. + KNO_3, 0.88 gm.
6	Check	A 6–6–5 (2% MgO) neutralized fertilizer (commercial)
7	N–P–Ca	NH_4NO_3, 0.77 gm. + $NH_4H_2PO_4$, 0.79 gm. + $Ca(NO_3)_2 \cdot 4H_2O$, 1.01 gm.
8	N–P–Mg	N and P (as above) + $Mg(NO_3)_2 \cdot 6H_2O$, 1.10 gm.
9	N–P–Na	N and P (as above) + $NaNO_3$, 0.74 gm.
10	N–P–K–Cl–SO_4	N and P (as above) + KCl, 0.32 gm. + K_2SO_4, 0.37 gm.
11	N–P–K–Ca	NH_4NO_3, 0.42 gm. + $NH_4H_2PO_4$, 0.79 gm. + KNO_3, 0.88 gm. + $Ca(NO_3)_2 \cdot 4H_2O$, 1.01 gm.
12	N–P–K–Mg	N and P (as above) + KNO_3, 0.88 gm. + $Mg(NO_3)_2 \cdot 6H_2O$, 1.10 gm.
13	N–P–K–Na	N, P, and K (as above) + $NaNO_3$, 0.74 gm.
14	N–P–Ca–Mg	N and P (as above) + $Ca(NO_3)_2 \cdot 4H_2O$, 1.01 gm. + $Mg(NO_3)_2 \cdot 6H_2O$, 1.10 gm.
15	N–P–Ca–Na	N, P, and Ca (as above) + $NaNO_3$, 0.74 gm.
16	N–P–Mg–Na	N and P (as above + $Mg(NO_3)_2 \cdot 6H_2O$, 1.10 gm. + $NaNO_3$, 0.74 gm.
17	N–P–K–Ca–Mg	NH_4NO_3, 0.08 gm. + $NH_4H_2PO_4$, 0.79 gm. + KNO_3, 0.88 gm. + $Ca(NO_3)_2 \cdot 4H_2O$, 1.01 gm. + $Mg(NO_3)_2 \cdot 6H_2O$, 1.10 gm.
18	N–P–K–Ca–Na	N, P, K, and Ca (as above) + $NaNO_3$, 0.74 gm.
19	N–P–K–Mg–Na	N, P, and K (as above) + $Mg(NO_3)_2 \cdot 6H_2O$, 1.10 gm. + $NaNO_3$, 0.74 gm.
20	N–P–Ca–Mg–Na	N and P (as above) + $Ca(NO_3)_2 \cdot 4H_2O$, 1.01 gm. + $Mg(NO_3)_2 \cdot 6H_2O$, 1.10 gm. + $NaNO_3$, 0.74 gm.
21	N–P–K–Ca–Mg–Na	NH_4NO_3, 0.27 gm. + $NaH_2PO_4 \cdot H_2O$, 0.94 gm. + $NaNO_3$, 0.15 gm. + $Ca(NO_3)_2 \cdot 4H_2O$, 1.01 gm. + $Mg(NO_3)_2 \cdot 6H_2O$, 1.10 gm. + KNO_3, 0.88 gm.
22	N–P–K–Ca–Mg–Na–Cl–CO_4	NH_4NO_3, 0.62 gm. + KCl, 0.32 gm. + K_2SO_4, 0.37 gm. + $NaH_2PO_4 \cdot H_2O$, 0.94 gm. + $Ca(NO_3)_2 \cdot 4H_2O$, 1.01 gm. + $Mg(NO_3)_2 \cdot 6H_2O$, 1.10 gm. + $NaNO_3$, 0.15 gm.

as previously reported (**4**), is adversely affected by mixtures of equivalent weights of K, Ca, Mg, and Na. Therefore, the results obtained with beans probably cannot be extended to apply generally to all crops.

Methods of evaluating the chemical analyses

The growth, expressed in terms of dry weight, and the K_2O, CaO, and MgO composition of the plants from all treatments except nos. 18, 19, and 20 are found in tables II and III. The treatments are arranged from left to right in the order of their influence on the total dry weight of foliage produced. All figures expressing the chemical composition are averages of duplicate determinations, made on duplicate samples in those cases where the material was sufficient to allow such a procedure. Determinations of nitrogen and phosphorus were made also, but were not included in order to simplify the tabular material and because in most cases the results indicated that nitrogen and phosphorus determinations were of no significance.

In discussing the absorption of the cations occurring in the plants fertilized with the different ionic combinations, as indicated by the figures in the tables, the percentage of the cation in the dried plant will be considered as a measure of the relative ability of the plant to utilize that material under the conditions of the experiment. The presence of a cation, expressed in terms of parts per million in the extracted sap of the stem of the freshly harvested plant, will be considered as reflecting the absorptive ability of the plant under each of the treatments, or as partially indicative of the availability of the cation to the plant. However, it also reflects the ability or inability of the plant to utilize the absorbed material when considered in relation to the total amount of a given cation that was absorbed. For example, a high content of a cation in p.p.m. in the stem sap may indicate rapid and unhindered absorption or may indicate an accumulation resulting from low total utilization. In turn, low total utilization may be considered as due to retarded growth or to the inability of the plant to build the particular cation into differentiated structures or substances. If the total utilization is not proportional to the growth made, the latter may be considered as the factor responsible for the low total utilization. From a comparative study of a cation in the plant in terms of percentage of the dry weight, p.p.m. in the stem sap, and total absorption per unit, it should be possible to determine whether or not the phenomena observed are due to the effect of the treatment on absorption, on utilization, or on both.

Absorption of cations

In order to identify and clarify some of the reasons for the differences in growth found (figs. 1, 2) resulting from the use of the N–P–K–Ca–Mg–Na treatment as compared with the N–P–K treatment, an outline is given

TABLE II

EFFECT OF CERTAIN CATIONS, USED SINGLY AND IN COMBINATION, ON THE GROWTH AND POTASSIUM, CALCIUM, AND MAGNESIUM ABSORPTION OF BEANS RECEIVING A UNIFORM NITROGEN AND PHOSPHORUS TREATMENT

TREATMENTS	5	8	9	7	14	13	16	12	15	11	17	21
N, P +	K	Mg	Na	Ca	Ca Mg	K Na	Mg Na	K Mg	Ca Na	K Ca	K–Ca Mg	K–Ca Mg–Na
Dry eight foilage (gm. per pot)	2.2	2.3	2.6	2.7	3.0	3.6	4.2	4.3	4.4	5.3	8.3	12.3
Potassium (K_2O):												
Percentage in foliage (dry weight basis)	5.9	2.0	1.9	2.0	2.0	4.9	1.7	5.6	1.7	5.2	4.6	4.0
P.p.m. in stem sap*	13500	2500	2000	3000	400	5200	800	6000	2000	6500	5700	4200
Mg. absorbed per pot	132	46	50	54	60	176	72	241	75	276	382	492
Percentage of application absorbed by plants	33	——	——	——	——	44	——	60	——	68	95	122
Calcium (CaO):												
Percentage in foliage (dry weight basis)	1.5	1.6	1.9	2.6	1.9	1.6	1.5	1.3	2.8	2.3	2.1	1.9
P.p.m. in stem sap*	300	375	650	1800	400	650	550	350	2000	750	650	400
Mg. absorbed per pot	33	37	50	70	57	58	63	56	123	122	174	234
Percentage of application absorbed by plants	——	——	——	29	24	——	——	——	51	51	72	97
Magnesium (MgO):												
Percentage in foliage (dry weight basis)	1.3	2.0	1.1	1.0	1.9	1.2	2.2	1.6	1.4	1.1	1.8	1.8
P.p.m. in stem sap*	50	650	900	1000	1100	110	125	200	650	125	600	525
Mg. absorbed per pot	29	46	29	27	57	43	93	69	62	58	149	222
Percentage of application absorbed by plants	——	28	——	——	35	——	57	42	——	——	92	136

* Soluble in a 2 per cent. acetic acid extract of the stems, and based on the fresh weight of the stem tissue.

in table II of the effects of each of the more significant combinations of the four cations on the K_2O, CaO, and MgO contents of the plants. In this series of treatments the nitrogen and phosphorus applications remained constant and equivalent weights of each cation were used in making up the different treatments. The first treatment (N–P–K) resulted in a larger accumulation of K_2O in the sap of the stems than was found in any of the other treatments. The addition of potassium had a more marked influence on potassium absorption than the addition of either calcium or magnesium had on their own absorptions. The potash content of the plant stem sap from the treatment in which potassium was the only cation used was 13,500 p.p.m. or over twice the amount found in the sap of plants in any other treatment. Probably this extremely large concentration of potassium was the factor that was responsible for the poor growth made with the N–P–K treatment. This amount of potassium probably was toxic, due to the fact that its presence in the plant sap in such a concentrated soluble proportion inhibited the absorption or utilization of sufficient calcium and magnesium to permit normal growth. Calcium was less detrimental to growth and absorption than any of the other cations used singly with nitrogen and phosphorus.

Of the six combinations of two cations, the K–Ca combination was most efficient in producing maximum growth and promoting maximum absorption of both potassium and calcium, but one of the least efficient in promoting the absorption of magnesium. The Ca–Mg treatment checked both absorption and utilization of potassium, as indicated by a low K_2O content in p.p.m. in the stem sap and by a low total potash absorption by the plant. The K–Na treatment resulted in a lower absorption and utilization of potassium than occurred in any of the other potassium treatments. In treatments numbered 9, 16, and 15, in which sodium was present, a low potassium absorption and utilization were also found. The K–Na combination was also detrimental to magnesium absorption and utilization. The Mg-Na treatment was detrimental to adequate absorption of potassium and magnesium and to the utilization of potassium and calcium. In this treatment the highest concentration of magnesium (2.2 per cent.) was found in the plant, but the absorption rate as indicated by a concentration of 125 p.p.m. on the stem sap was checked by some external factor, probably a lack of antagonism between the magnesium and sodium ions (7). In the K–Mg combination the low absorption and utilization of calcium probably is most responsible for the resultant lack of growth, but the detrimental influence of potassium on magnesium absorption as observed in the N–P–K treatment probably also existed. In the Ca–Na combinations the antagonistic effect of the sodium ion on the calcium ion resulted in a greater absorption of calcium, indicated by a concentration of 2000 p.p.m. of CaO in the stems, and also in a greater utilization, indicated by a CaO content of 2.8 per cent. of the dry weight of the plant. However,

the detrimental influence of sodium on potassium absorption overshadows any beneficial effect of calcium on potassium absorption, and results in a low potassium absorption, which probably was the factor responsible for poor growth. The K–Ca combination was the best double cation combination used, because Ca did not exert a detrimental effect on potash absorption and overcame the detrimental effect of potassium on calcium absorption. The nutrient limiting growth in this treatment was probably magnesium.

The addition of magnesium to the K–Ca combination increased magnesium absorption to a satisfactory degree and, because its detrimental influence upon potassium and calcium absorption was held in check by the inclusion of these two ions, resulted in satisfactory growth.

The addition of the sodium ion to the K–Ca–Mg combination necessitated the use of $NaH_2PO_4 \cdot H_2O$ instead of $NH_4H_2PO_4$ in order that the nitrogen content could be maintained at a constant level for all treatments. The increase in growth produced with this combination over that produced with the K–Ca–Mg treatment was not associated with any beneficial effects on the absorption of potassium, calcium, or magnesium. As the phosphorus content was increased from 0.52 to 0.65 per cent. of the dry weight of the plant by including the sodium cation, increased phosphorus availability and utilization were probably responsible for the increased growth produced. Whether or not the sodium ion as such, or the form in which the phosphorus ion was used, was responsible for the increased phosphorus absorption, was not determined. However, in a previous work (**4**) sodium, when used in the same formulation with K–Ca–Mg, was very detrimental to the growth of potatoes. Hence it would seem, from a comparison of the behavior of potatoes and beans, that beans are the more tolerant of the sodium cation when it is associated with the other cations.

Discussion of cation absorption

In beans, the potassium cation, when added to the soil, produced such a dominant effect on potassium absorption that it was able to counteract the detrimental effect of any of the other cations on its absorption by the plant. However, neither the calcium nor the magnesium cations were able to exert such a dominant effect on their own absorptions when they were combined with other cations that tended to interfere with their own absorptions.

The concentration of potassium in the stems of the plants indicated a high degree of negative correlation with growth. For example, treatments numbered 5, 13, 12, 11, 17, and 21 had all received an equal quantity of potassium and the stem sap of the plants from these treatments ranged in p.p.m. of K_2O from 13,500, in the case of those exhibiting the poorest growth, down to 4200 p.p.m., in the plants exhibiting the best growth. From these observed phenomena we may be safe in concluding that nothing interfered with potas-

sium utilization or absorption to any marked extent. Had this not been the case, the concentration of K_2O in the sap would have dropped below the optimum with the increased utilization or lack of absorption of potassium by the plant, or, would have increased above that found at minimum growth.

Sodium had a marked stimulating effect on calcium absorption, while potassium exerted little or no influence on calcium absorption and magnesium had a marked detrimental effect on the absorption of this cation. These influences were quite evident when the CaO content of the sap from treatments 7, 14, 15, 11, 17, and 21 were compared. In the Ca treatment 1800 p.p.m. of CaO was found in the stem sap. However, when magnesium was added, the CaO content of the sap dropped to 400 p.p.m. although growth did not increase more than 10 per cent. When sodium was substituted for magnesium, the CaO content of the sap increased to 2000 p.p.m. under conditions in which the growth also increased over 50 per cent. However, as the CaO content of the sap from the plants of treatments 11, 17, and 21 showed some indication of negative correlation with growth, it is evident that potassium when applied in conjunction with calcium was able to minimize the negative influence of magnesium and the positive influence of sodium on calcium absorption.

Potassium, calcium, or sodium had a greater influence on magnesium absorption by the plant when used in conjunction with magnesium than the magnesium cation had on its own absorption. Although magnesium was detrimental to the absorption of calcium, calcium facilitated magnesium absorption and utilization. This phenomenon may be partly responsible for the fact that dolomitic limestones are generally as efficient correctives for a condition of magnesium deficiency in the soil and plant as the more soluble magnesium salts (**3**). Both sodium and potassium prevented normal magnesium absorption and potassium interfered also with its utilization in the plant. These phenomena were evident when the magnesium contents of the plants in treatments 8, 14, 16, 12, 17, and 21 were compared. With magnesium alone, the MgO content of the stem sap was 650 p.p.m., but when calcium was added it increased to a probable toxic concentration of 1100 p.p.m. even though a 25 per cent. increase in growth was made. The Ca–Mg combination was found to be toxic to the potato plant also (**4**), manifested by a purpling of the central portions of the leaf. However, when sodium was associated with magnesium, the MgO content of the sap fell to 125 p.p.m. This phenomenon probably was due to a lack of absorption brought about by a lack of antagonism. MILLER (**7**) reports that an antagonistic influence between the sodium and magnesium ions has not been demonstrated. This phenomenon may be due in part, however, to the rapid utilization of magnesium. Again, when potassium was associated with magnesium, the MgO content of sap dropped to 200 p.p.m. However, when calcium was associated

with the K–Mg combination, its beneficial influence on absorption counteracted the detrimental effect of potassium, and both normal absorption and normal utilization resulted.

Effect of various anions on cation absorption

Although the various cations exerted marked influences on growth, as indicated by a difference of 10.1 gm. in the dry weight produced per pot between the N–P–K and the N–P–K–Ca–Mg–Na treatments, some of the anions were also of physiological significance, as shown by the analyses in table III.

The use of nitrogen alone (treatment no. 2) did not depress calcium or magnesium absorption below that occurring under optimum growth conditions. However, a slight depression in potassium absorption and a large increase in nitrogen absorption (4540 p.p.m. of soluble N) as compared with a nitrogen absorption under optimum growth conditions of 830 p.p.m. indicated that the concentration of these two materials probably was responsible for the poor growth. Additions of either PO_4 or K to the NO_3 treatment depressed Mg absorption to a point where it became one of the growth-limiting factors. Potassium with nitrogen increased potash absorption to a concentration where it again probably caused the previously mentioned unbalanced or toxic condition in the plant. In the unfertilized treatment a striking increase in the absorption of calcium and magnesium over that of the previously discussed treatments occurred, but the absorption of potassium fell below that for the plants in the other treatments. Poor growth in the unfertilized pots probably was due chiefly to a lack of nitrogen as indicated by a concentration of 81 p.p.m. of soluble N in the sap of the stems. However, it was observed in the N–K treatment that by the addition of nitrogen and potassium alone growth was depressed below that found when no fertilizer was used, which would seem to indicate that any fertilizer applied must have a rather definite ionic balance.

The commercial fertilizer treatment produced a larger amount of dry weight than any of the combinations of N, P, or K, but it was materially smaller than that produced with the N–P–K–Ca–Mg–Na treatment (no. 21). The absorption and utilization of both potassium and calcium approximated that found in the N–P–K–Ca–Mg–Na treated plants, but the decreased utilization of magnesium, as shown by a magnesia composition of 1.2 per cent. as compared with 1.8 per cent. in the optimum treatment, indicated that some phenomenon was still hampering the normal functioning of the plant.

After comparing the dry weight produced in treatment no. 21 with that of no. 22, it must be concluded that either or both chlorine and sulphate ions were partially responsible for the poor growth realized with a commercial fertilizer treatment containing large amounts of both of these ions. A toxic

TABLE III

EFFECT OF CERTAIN IONS ON THE GROWTH AND POTASSIUM, CALCIUM, AND MAGNESIUM ABSORPTION OF BEANS

TREATMENTS	2 N	3 N–P	4 N–K	1 NOTHING	5 N–P–K	10 N–P–K Cl–SO_4	6 COMMERCIAL	22 N–P–K Ca–Mg–Na Cl–SO_4	21 N–P–K Ca–Mg Na
Dry weight foliage (gm. per pot)	1.0	1.7	1.8	2.1	2.2	2.9	4.6	6.8	12.3
Potassium (K_2O):									
Percentage in foliage (dry weight basis)	2.4	2.2	6.3	2.2	5.9	6.1	4.9	5.5	4.0
P.p.m. in stem sap	3000	2500	11000	1800	13500	12000	4500	4000	4200
Mg. absorbed per pot	24	36	113	46	130	177	225	374	494
Percentage of application absorbed by plants	——	——	28	——	32	44	56	93	122
Calcium (CaO):									
Percentage in foliage (dry weight basis)	2.0	1.7	1.3	3.4	1.5	1.3	2.2	1.9	1.9
P.p.m. in stem sap	400	650	350	3200	300	400	900	400	400
Mg. absorbed per pot	20	29	23	72	33	37	101	129	234
Percentage of application absorbed by plants	——	——	——	——	——	——	——	53	97
Magnesium (MgO):									
Percentage in foliage (dry weight basis)	1.2	1.2	1.2	1.4	1.3	0.9	1.2	1.9	1.8
P.p.m. in stem sap	500	125	30	1500	50	40	550	425	525
Mg. absorbed per pot	12	20	22	29	29	26	55	129	222
Percentage of application absorbed by plants	——	——	——	——	——	——	——	79	136

or unbalanced anion condition resulting from the presence of Cl or SO_4 ions in treatment no. 22 is responsible for the difference in the growth produced between treatments 22 and 21, because the potassium, calcium, and magnesium utilization and absorption in both treatments were fairly comparable. In treatment 10 in which Cl and SO_4 ions were added to the N–P–K treatment, an increased growth was realized. The fact that both had considerably less total dry weight than the N–P–K–Ca–Mg–Na treatment indicated that in this comparison some other factor or factors were more detrimental to growth than the presence in the soil of either Cl or SO_4. In fact, the addition of Cl and SO_4 ions to the N–P–K mixture caused a slight increase in plant growth, which indicated that these two ions are of value in partially counteracting a certain type of unbalance, but that after this condition is corrected by the addition of Ca, Mg, and Na ions, their own toxic influence becomes one of the factors limiting the growth of beans.

Summary of the influence of various ions on cation absorption and utilization

The influences of most of the common ions included in a normal mixed fertilizer on the absorption and utilization of the three cations, potassium, calcium, and magnesium, by bean plants, are summarized in schematic form in table IV. In this summary the mean influence of the ions in all combinations, considered in relation to the optimum treatment N–P–K–Ca–Mg–Na,

TABLE IV

Summary of the influence of various ions on cation absorption and utilization by beans

Ion	NO_3 NH_4	PO_4	Cl SO_4	K	Ca	Mg	Na
K_2O absorption	–	–	0	+++	0	––	–
K_2O utilization	–	–	0	+++	0	–	––
CaO absorption	0	0	0	–	++	––	+++
CaO utilization	0	0	0	–	++	–	++
MgO absorption	0	–	0	–––	+++	0	––
MgO utilization	–	–	0	––	+	0	+

Key: ––– very detrimental (probably resulting in a deficiency); –– detrimental; – slightly detrimental; 0 probably of no influence; + slightly beneficial; ++ beneficial; +++ extremely beneficial (probably resulting in a toxicity).

was used as a basis in estimating the effects listed. As mentioned earlier in the paper, 15.9 gm. of replaceable K_2O, 2.8 gm. of replaceable CaO, and 1.25 gm. of replaceable MgO per pot were found in the unfertilized soils. Some differences in the influence of each ion would probably be observed under a different condition of base saturation. However, as the bean crop was the third and last of a series grown on this soil medium, very likely only small

portions of the total exchangeable cations were influencing actively the phenomena observed. This contention would seem to be substantiated by the fact that on the unfertilized soil 15.9 gm. of replaceable K_2O had no apparent detrimental influence on calcium and magnesium absorption, while the addition of 0.40 gm. of K_2O in a soluble form had a most detrimental effect on the absorption of these two ions.

General summary and conclusions

1. The present trend in the fertilizer industry toward the elimination of sodium, chlorine, and sulphur, a reduction in the amount of calcium, and the inclusion of magnesium in increasing amounts, warranted a study of the effects of these materials on growth, cation absorption, and utilization.

2. On a soil medium that in the past had received considerable fertilizer under field conditions, bean plants without additional fertilizers were able to absorb sufficient calcium and magnesium, but did not make a normal growth, probably because of a lack of nitrogen, phosphorus, and potash.

3. When an N–P–K mixture was added to the above mentioned unfertilized soil the intake of magnesium and calcium was depressed to such an extent that they became two of the limiting factors to optimum growth.

4. The addition of the potassium cation to a soil stimulated the potash absorption by bean plants to a greater extent than the addition of calcium or magnesium cations stimulated their absorptions by the plant. None of the other cations employed had any very pronounced beneficial or detrimental influence on potassium absorption when the potassium ion was included in the fertilizer treatment. However, the addition of potassium when combined with magnesium materially reduced the absorption of both calcium and magnesium, and when combined with calcium reduced magnesium absorption. It did not effect an increased absorption of any other ion.

5. The addition of calcium to a soil not only increased calcium absorption, but in all cases facilitated a large magnesium absorption, and in some cases was slightly beneficial to potassium absorption.

6. The absorption of the magnesium cation by bean plants was the most difficult phenomenon to control, because the addition of the magnesium cation to a soil had less influence than that of any of the other cations used on magnesium absorption.

7. The sodium ion had a beneficial effect on calcium absorption and utilization, and was beneficial to magnesium utilization, but in most cases was slightly detrimental to the absorption of potassium and extremely detrimental to the absorption of magnesium.

8. The only method of obtaining an adequate absorption of all three cations was to include them in the fertilizer mixture. Under these conditions the addition of the sodium ion to this combination was not detrimental to the growth of beans and possibly was of some direct benefit.

9. The anions NO_3, PO_4, Cl, and SO_4 had no detrimental influence on

calcium intake and utilization, but the NO_3 and PO_4 anions had a slight detrimental effect on potassium absorption and probably a slight detrimental effect on magnesium utilization.

10. The use of rapid chemical methods in analyzing the sap of plant stems was found to be of value in determining whether deficiencies of the cations were due to a lack of absorption or to a lack of utilization by the plant.

11. From this work it may be concluded that if plants showing *poor* growth have a higher content of a given nutrient (as determined by p.p.m. in stem sap) than similar plants showing a *superior* growth, something is interfering with the proper utilization of that nutrient *after* its absorption by the plant. This is the situation in the potassium series. However, if the plant sap of plants of *superior* growth has a higher content of a given nutrient than that of plants showing *poor* growth, the cause is likely to be lack of ability to absorb this nutrient in the case of the stunted plants. This occurs in the magnesium series. Failure to absorb may be due to a deficiency of the nutrient in an available state, or to some absorption phenomenon such as lack of antagonism.

12. The results in these tests should not be construed as applying to all plants, as work not far enough advanced to report at this time has indicated that other plants do not respond in a manner similar to that herein reported for beans.

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CHEMICAL AND SPECTROSCOPIC ANALYSIS OF PHLOEM EXUDATE AND PARENCHYMA SAP FROM SEVERAL SPECIES OF PLANTS

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(WITH THREE FIGURES)

Introduction

A problem which has occupied the attention of botanists for many years is that of the transport of organic and inorganic substances in plants particularly with respect to the tissues active in translocation. Since there is still some doubt concerning the transport of inorganic constituents in the phloem, it would seem desirable in this connection to make an analysis of phloem exudate to see what materials may be present therein. The exudate used in these analyses is probably restricted to phloem tissues alone as methods of collection were used which have been employed by previous investigators (HARTIG, **12,** MÜNCH, **20,** and CRAFTS, **3**). JAMES and BAKER (**15**), in working with maple claim that the sap exuded did not come from the phloem alone but also from the living cells particularly of the cambial zone. Their work, however, was done in England during the winter months when the cambial activity must have been slight. GROSSENBACHER (**10**), EAMES and MACDANIELS (**7**) and other investigators have abundant evidence to indicate that the maximum cambial activity occurs at the time of elongation of the shoots which is in the early part of the summer for the species included in this study. Hence the chance of exudation from the cambium along with the phloem exudation is slight, since the exudate for the present study was collected during the months of August and September. In this connection PRIESTLEY (**23**) reports that if the bark is stripped from the new wood in May or June and a cut made across the differentiating tissues of soft wood, a fine spray of liquid spurts several inches from the cut while a similar cut in hard wood results in a gentle oozing with no spray. These observations tend to support the idea that exudation is not limited to the phloem alone during the active growing season. They do not indicate, however, that the exudate later in the season should be from tissues other than the phloem.

PFEIFFER (**22**) has made determinations on the osmotic concentrations of sieve tube sap of *Quercus rubra* by a cryoscopic method and finds a range of values from 15.0 atm. in August to 21.1 atm. in November. MÜNCH (**20**) gives values for osmotic pressure of sieve tube sap of *Quercus rubra* varying from 20.9 atm. to 23.7 atm. and values from 25.8 atm. to 34.3 atm. for sieve tube sap of *Robinia pseudo-acacia.*

KRAUS (**16**) and ZACHARIAS (**26**) have made some qualitative tests on the

sieve tube sap of *Cucurbita pepo*. A quantitative analysis of phloem exudate has not been made, however, previous to this work probably because of the difficulties attendant upon collection of sufficient quantities of the exudate to permit an analysis by methods other than microchemical or spectroscopic. It has been the purpose of this work to analyze quantitatively the phloem exudate and parenchyma sap with respect to the following: pH, total sugars, reducing sugars, calcium, magnesium, potassium, lithium, nitrate nitrogen, and total nitrogen, and to make a qualitative spectroscopic analysis for any other inorganic elements that might be present in the sample. The species studied were *Fraxinus americana, Fraxinus pennsylvanica* var. *lanceolata, Platanus occidentalis, Robinia pseudo-acacia,* and *Cucurbita maxima*. Quantitative spectroscopic methods of analysis which will be described later were used wherever possible because of their efficacy in dealing with small amounts of material.

The method of collecting the phloem exudate in the woody plants is one that has been used by MÜNCH (**20**). A slit in the trunk about two inches long at an angle of about 45° with the horizontal made with a sharp knife was found to yield the best exudation. The cut was made into the active phloem tissues from which would exude amounts of sap varying from a few drops to 2 or 3 cc. depending upon the species and the length of the cut. As the drops of liquid appeared, usually near the base of the cut, they were picked up with a glass capillary tube fitted with a small rubber bulb and transferred immediately to hard glass phials which contained a few cc. of toluene as a preservative. Several cuts were made at a time and the amount usually collected after two or three hours was about 10 cc.

The time of day seemed to be a factor in governing the amount of flow, the largest quantities being obtained in the afternoon. Bright sunny days usually gave better results than cloudy cold ones, although on a few occasions the flow from *Robinia pseudo-acacia* was good on cloudy days. On days when the transpiration stream was rapid a cut deep enough to penetrate to the xylem tissue would result in the exudate being sucked into the transpiration stream often with an audible sucking sound.

HARTIG (**12**) and MÜNCH (**20**) report that a second cut within from one to five meters below, and for a shorter distance above, an original cut would yield no flow. However, in both species of *Fraxinus,* and in *Platanus occidentalis* the presence of a second cut either above or below the first cut did not appreciably affect the flow from the first cut unless the second cut was within 5 or 6 cm. on either side of the first. The flow from the second cut would then be as good as that from the first. The second cut would give a poor flow when too close to a cut above, and a second cut would diminish the flow from the first cut when too close above the first. A distance of 10 or 12 cm., however, seemed to make no difference except in *Robinia pseudo-acacia*

where a second cut would yield no flow unless displaced laterally with respect to the first. In this species a second cut even 1 or 2 meters directly above or below a first cut would yield no flow.

In the case of *Cucurbita maxima* the method of collection of phloem exudate was that used by CRAFTS (3). The plants were grown in the greenhouse, and when the fruits had developed to a length of about 8 or 10 cm. they were cut from the vine leaving about 2.5 cm. of peduncle attached to the fruit. A drop of crystal clear exudate coming from the phloem proper and from the ectocyclic and entocyclic sieve tubes which are in the position of a latex system would appear on this cut end of the peduncle. This was allowed to drop into a small porcelain crucible containing a few cc. of toluene. About two drops of exudate would be obtained before the flow stopped, but the flow could be started again by cutting a short section from the end of the peduncle. This process could be repeated until the peduncle had been cut back to the fruit and a total of about ten drops of exudate obtained. Fifteen or twenty fruits would give a sample sufficient for several of the analyses.

The parenchyma sap was obtained by freezing, at $-15°$ C., leaf tissue contained in large test tubes in an alcohol bath cooled by a G. E. cooling unit, and then pressing out the sap by means of an hydraulic press. Pressures of 10,000 pounds per square inch were used, and the tissue was subjected to this pressure for five minutes. Samples of the sap were kept in the refrigerator until ready for use.

Analytical methods

The pH values of all samples were obtained immediately after collection by use of the quinhydrone electrode method described in Leeds-Northrup Co., Bulletin no. 3.

The sugar analyses were made as soon after collection as convenient, in order to minimize the error due to chemical changes. All samples were shaken well with toluene and kept in the refrigerator until analysis was begun. The toluene prevented any changes due to the growth of organisms but had no effect on any enzymes which might have been present in the exudate. It is not certainly known that any enzymes were present. The modified SHAFFER (**24**) method of sugar analysis was used since the colorimetric determination of the Cu_2O is very accurate when small quantities of material must be used.

The modification of the spectroscopic method for the determination of potassium as developed by HICKS (**13**) will be described in some detail. The absence of sensitive arc lines of potassium when small amounts of the element are involved prevents the use of the arc and spectrograph in quantitative analyses for this element; but there is a line in the flame spectrum of potassium (wave length 7669 Å.) which is extremely sensitive and which shows

up when the element is present in small quantities. The duration of this line when potassium is introduced into the flame is proportional within limits to the amount of the element present in the flame. The duration of the line is measured by direct visual observation, the observer starting a stop watch upon appearance of the line and stopping the watch upon the line's disappearance. The line flashes into view suddenly and fades out as rapidly so that the timing can be accurate to within 0.1 second. The spectrometer used was the ordinary three arm type described by BALY (1). The Bunsen flame of illuminating gas was standardized as far as possible as to position with respect to the slit of the instrument, height, and mixture. The substance was introduced into the flame in the following manner. A platinum wire was coiled into a small spiral of five turns, 2 mm. in diameter, in such a way that the turns of wire were not in contact in any part of the coil. This coil, once wound, was not disturbed in shape or size during the entire series of tests, and when it was dipped into a liquid it would always pick up and hold the same size drop thus assuring the same amount of a given concentration of material in the flame each time an observation was made. After the platinum wire was dipped into the solution it was held near another flame until the liquid had evaporated leaving only a solid incrustation on the wire which was then introduced into the flame and observed. This evaporation prevented loss of material due to spattering, which would result if a drop of liquid were suddenly thrust into the Bunsen flame.

Standard solutions of potassium chlorate[1] containing potassium in amounts of 0.32, 0.64, 0.96, 1.28 mg./cc. were made from recrystallized salt (Baker's analyzed chemicals). The duration of the 7669 Å. line was observed for each of these concentrations, the average time of ten observations in each case being taken as the final result.

TABLE I

RELATION BETWEEN CONCENTRATION OF SOLUTION AND DURATION OF THE POTASSIUM LINE 7669 Å.

TIME	CONCENTRATION OF POTASSIUM
sec.	*mg/cc*
2.00	0.16
2.40	0.32
2.60	0.48
2.80	0.64
3.00	0.80
3.30	0.96
3.72	1.28

[1] It was later learned that the use of perchloric acid would have given more accurate results, with potassium chloride rather than potassium chlorate taken as standard.

Table I shows the results of such a series of observations and figure 1 shows the curve of concentration of solution plotted against duration of line.

It is necessary now to observe the duration of line 7669 Å. for the various samples of exudate and sap and from the observed times read from the curve the corresponding values of the concentration of potassium. Because the presence of sugars and other organic materials in the exudate prevented the

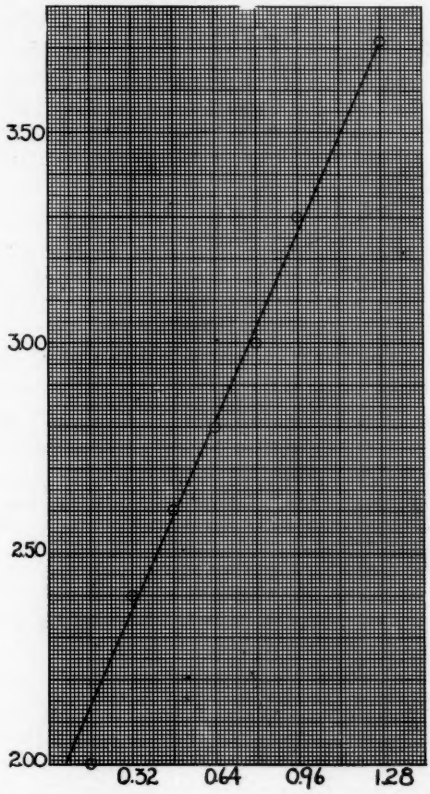

FIG. 1. Relation between concentration of solution and duration of potassium line 7669 Å.

use of the material directly, a known volume of the exudate was evaporated to dryness and ashed in a muffle furnace at a temperature not exceeding 450° C. The temperature was kept comparatively low in order to prevent any loss of the calcium, magnesium or potassium compounds during the ashing process. The ash was dissolved in hydrochloric acid[2] and brought

[2] See footnote p. 368.

back to the original volume with distilled water. This solution was then given five tests and the average time for the five taken as final. Since these values seldom varied by more than 0.2 sec., five observations were considered sufficient. The corresponding concentrations for each sample were recorded. During a series of observations on unknowns the system was checked from time to time by making observations on one or more known concentrations, and at the beginning of a new series of observations at a later date the system was brought up to the same standards or a new series of standards was set up.

The method of analysis for lithium was carried out in the same manner, the wave length of the line observed being 6709 Å. Curves of time *vs.* concentration were drawn and the concentrations read quantitatively wherever possible. Since the amounts of lithium were very minute in all species except *Platanus occidentalis* the analysis is really quantitative for that species only.

The spectroscopic method used for calcium and magnesium has been described by several workers, namely, LEWIS (**18**), GERLACH (**9**), SMITH (**25**), and HOAG (**14**). The spectrograph (Bausch and Lomb Optical Co., Rochester, N. Y.) was equipped with a quartz optical system of the very best type and a photographic plate holder for taking $4'' \times 10''$ spectrograms.

Electrodes of graphite (Acheson Graphite Co., Niagara Falls, N. Y.) were employed. These were arced for 30 seconds before using in order to burn out any impurities, particularly the calcium and magnesium present.

There are many lines present in the arc spectrum of calcium and magnesium when these elements are present in the arc in fairly large amounts. The intensity of these lines depends upon the length of exposure, the power used by the arc, the characteristics of the instrument, the characteristics of the plate, and the time of development. However, as the concentration of these elements in the arc is decreased, the number of lines visible when other factors are held as constant as possible decreases until only the so called "raies ultimes" or ultimate lines (DEGRAMONT, **5**) are present. These ultimate lines become less and less intense as the concentration of the substance in the arc decreases, the less sensitive lines fading out completely and the more sensitive lines remaining as the dilution is carried still further. Finally a point is reached at which the last of the lines fail to show in the spectrogram, and the presence of the element can no longer be detected at this or lower concentrations.

In order to make this method quantitative it is necessary to make spectrograms of known concentrations of the element in question, of the same order of magnitude as in the unknown, and to standardize the intensities of certain lines with respect to concentration. Two methods of procedure may be followed in this respect. In the first, the intensities of the spectral lines are measured as a function of the density of the line on the photographic plate

by a MOLL (19) recording microphotometer. This instrument provides a beam of light supplied by a bulb maintained at constant voltage which is focused on the line on the plate. The light transmitted by the line passes through a slit and falls upon a thermopile which is connected to a sensitive galvanometer, the mirror of which throws a light beam on a scale. The

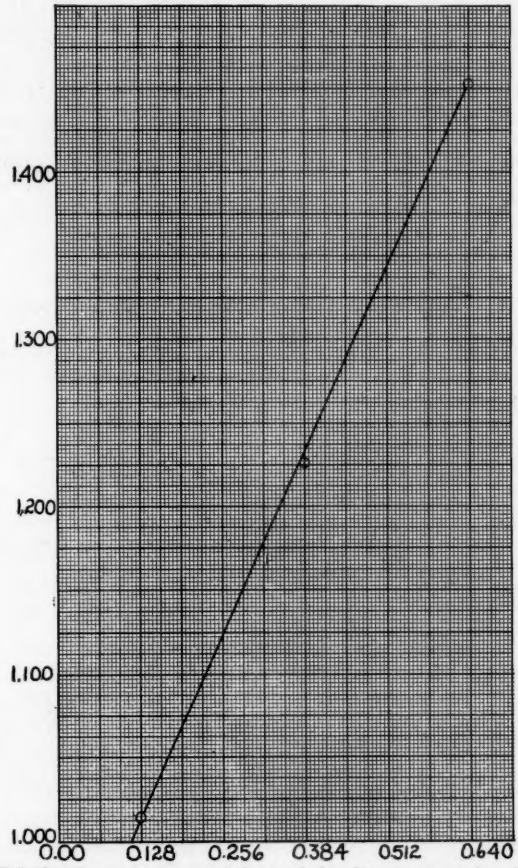

FIG. 2. Relation between concentration and density of magnesium line 2802 Å.

density of the line in question is taken by definition to be the $\log_{10}$ of the ratio of the light incident upon the line to the light transmitted by the line. This ratio is obtained by taking a reading of the galvanometer with the light shining through the clear plate on either side of the line, which gives the maximum amount of light transmitted by the plate (M. T.). A reading is

taken with the light shut off to find the point of maximum blackness (M. B.). The plate is then moved into position so that the light shines directly through the line in question and the reading of the galvanometer is again taken (P.). The ratio is then expressed as $\log_{10} \frac{\text{M. T.} - \text{M. B.}}{\text{P.} - \text{M. B.}}$. Table II and figure 2 show the relation existing between concentration of element in the arc and density of the line produced.

TABLE II

RELATION BETWEEN CONCENTRATION AND DENSITY OF MAGNESIUM LINE 2802 Å.

CONCENTRATION OF MAGNESIUM	DENSITY $\text{Log}_{10} \frac{\text{M. T} - \text{M. B.}}{\text{P.} - \text{M. B.}}$
mg/cc	
0.128	1.0133
0.384	1.2263
0.640	1.4523

Duplicate spectrograms of three different known concentrations are made on each plate along with duplicate exposures of seven samples of the material to be analyzed. The curve is drawn and the unknown concentrations are read from it.

The second method of determining the concentration of the solution in the arc consists of observing visually the concentrations at which certain lines are just visible and making comparison between the known concentrations and the unknowns. This is a good way to check the photometer method, but is not reliable enough to be employed as the sole method except when used by one long accustomed to such work. In this work all plates were read on the microphotometer and checked by the second method as well.

The substance to be analyzed was introduced into the arc in the following manner. A drop (volume 0.02 cc.) of the solution of the ash was placed on the hollowed out top of the lower electrode while it was still warm from the current which had burned out the impurities. The drop evaporated leaving a solid incrustation on the electrode always of the same quantity of material provided the concentration was kept constant. The arc was struck. The exposures were ten seconds in length, and the current was maintained at ten amperes from a 110 volt. D. C. line. Fresh electrodes were then introduced into the arc and other exposures made.

In order to rule out as far as possible errors resulting from plate differences and development, a series of known concentrations were run for each plate of unknowns and the concentrations of the unknowns read from the curve drawn for each individual plate.

Table III and figure 3 show the relation between concentration and density of line for calcium (wave length 3968 Å.).

TABLE III

RELATION BETWEEN CONCENTRATION AND DENSITY OF CALCIUM LINE 3968 Å

CONCENTRATION OF CALCIUM	DENSITY $\text{Log}_{10} \frac{\text{M. T.} - \text{M. B.}}{\text{P.} - \text{M. B.}}$
mg/cc	
0.18	1.0133
0.54	1.1139
0.90	1.2180

The accuracy of the spectroscopic method of analysis for calcium, magnesium, and potassium was checked by ordinary analytical methods recommended by "Official Methods" (1930). The spectroscopic analyses for calcium and magnesium were found to vary by not more than 5 per cent. and for potassium by not more than 8 to 10 per cent.

Because the elements boron, copper, manganese, and strontium did not show up on the plates when the solutions of the ash were arced at their natural concentrations samples of material were ashed and then made up to much smaller volumes of solution of ash which concentrated the material to such an extent that qualitative analyses could be made for any of the rarer constituents.

The Gunning method modified to include the nitrogen of nitrates (**21**) gave the results on total nitrogen, and the nitrate nitrogen determination was made by a modification by FREAR (**8**), for use with plant juice, of the phenoldisulphonic acid method of HARPER (**11**).

Identical methods were used for the treatment and analysis of the parenchyma sap which was collected during June of the following year. It was thought desirable to make these analyses on parenchyma sap in order to compare its composition with that of the phloem exudate from the identical trees.

Results

Table IV shows the results of analyses of phloem exudate of seven trees of *F. americana*. It is noted that the pH value is considerably higher than the pH of the parenchyma sap as shown in table VII. Appreciable amounts of calcium, magnesium, and potassium are present in all cases while a trace of lithium is noted. The amount of reducing sugar is much smaller than sucrose except in no. 5, and the large quantities of sucrose present should be noted. Sufficient exudate was obtained in two cases only for the nitrogen determination. The amount of total nitrogen present is much greater than

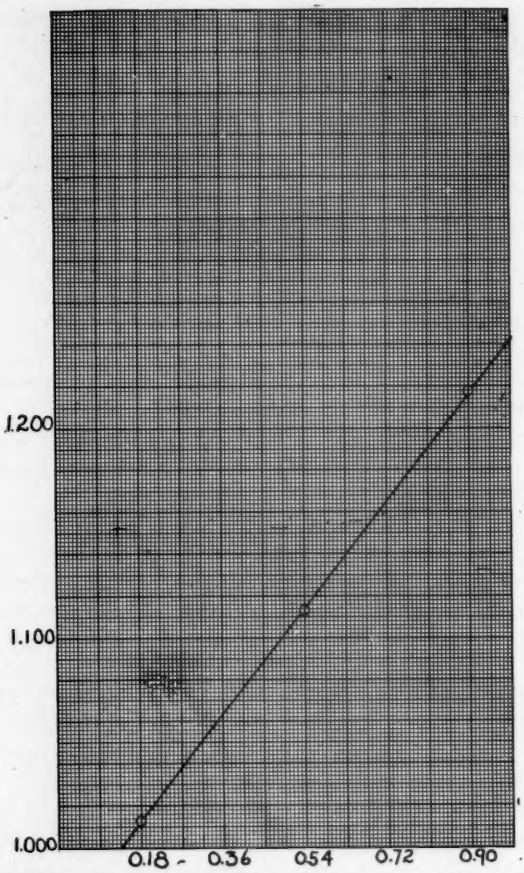

FIG. 3. Relation between concentration and density of calcium line 3968 A.

the total nitrogen in the phloem exudate of any other species studied. Also there is an appreciable amount of nitrate nitrogen present.

Results in table V show that the phloem exudate of the two species included in it have rather high pH values, appreciable amounts of calcium, magnesium, and potassium, and a trace of lithium. The reducing sugars are consistently low, but the sucrose is exceptionally high, particularly in *Robinia pseudo-acacia*. Nitrate nitrogen also is present.

It is evident from table VI that the phloem exudate of *Platanus occidentalis* has larger amounts of lithium than any of the other species studied, and that the amount of reducing sugar is greatly increased in absolute

TABLE IV

PHLOEM EXUDATE ANALYSIS OF *Fraxinus americana*

	DATE							
	SEPT. 2	SEPT. 20	SEPT. 10	AUG. 1	AUG. 1	AUG. 5	AUG. 15	AUG. 15
TREE NO.	1	2	3	4	5	6	7	Av.
pH	7.15	7.07	7.30	7.05	7.20	7.49	7.25	7.22
Ca *mg/cc*	1.18	1.09	1.04	0.55	0.76	0.86	0.34	0.83
Mg *mg/cc*	0.53	0.21	0.44	0.18	0.32	0.36	0.24	0.33
K *mg/cc*	0.99	0.45	0.64	1.20	1.02	0.58	1.12	0.86
Li *mg/cc*	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
REDUCING SUGARS *mg/cc*	11.12	Trace	0.84	0.22	30.18	2.64	1.98	7.83
SUCROSE *mg/cc*	72.64	70.54	77.40	102.18	65.46	93.71	86.45	81.20
TOTAL SUGARS *mg/cc*	83.76	70.55	78.24	102.4	98.64	96.35	88.43	88.34
NITRATE NITROGEN *mg/cc*		0.11	0.095					0.103
TOTAL NITROGEN *mg/cc*		2.45	3.64					3.05

TABLE V

PHLOEM EXUDATE ANALYSIS

	Fraxinus pennsylvanica var. *lanceolata*		*Robinia pseudo-acacia*			
	DATE		DATE			
	SEPT. 1	AUG. 20	SEPT. 1	SEPT. 15	AUG. 5	AUG. 5
TREE NO.	1	2	1	2	3	4
pH	7.58	7.20	7.4	7.36	7.23	7.25
Ca *mg/cc*	1.04	1.33	0.90	0.44	0.72	0.72
Mg *mg/cc*	0.61	0.21	0.38	0.15	0.39	0.38
K *mg/cc*	0.37	0.58	1.14	1.21	0.90	0.95
Li *mg/cc*	Trace	Trace	Trace	Trace	Trace	Trace
REDUCING SUGARS *mg/cc*	3.41	3.81	2.08	4.78		0.51
SUCROSE *mg/cc*	107.99	118.09	177.82	147.94		199.94
TOTAL SUGARS *mg/cc*	111.4	121.9	179.9	152.72		200.45
NITRATE NITROGEN *mg/cc*	0.12					0.135
TOTAL NITROGEN *mg/cc*	0.49					0.56

TABLE VI

PHLOEM EXUDATE ANALYSIS

	Platanus occidentalis				*Cucurbita maxima*	
	DATE				DATE	
	SEPT. 20	AUG. 30	AUG. 30	SEPT. 10	FEB.	FEB.
TREE NO.	1	2	3	4	1	2
pH	7.35	7.31	7.28	7.31	8.1	8.0
Ca *mg/cc*	1.26	1.26	0.76	0.84	0.18	0.07
Mg *mg/cc*	0.66	0.24	0.28	0.19	0.06	0.10
K *mg/cc*	0.96	0.80	0.90	0.90	0.24	0.20
Li *mg/cc*	0.08	0.04	0.08	0.158	None	None
REDUCING SUGARS *mg/cc*	86.4	67.3		56.5	4.83	2.07
SUCROSE *mg/cc*	1.90	40.3		48.4	2.83	3.33
TOTAL SUGARS *mg/cc*	88.30	107.6		104.9	7.66	5.4
NITRATE NITROGEN *mg/cc*	0.203					
TOTAL NITROGEN *mg/cc*	0.28					

amounts and also in ratio to the total sugars. This increase in reducing sugar may be due in part to the change of sucrose to reducing sugar during the interval between collection of the exudate and analysis. The use of toluene as a preservative, while preventing the growth of organisms in the exudate, would not hinder the action of any enzymes which might cause inversion of the sucrose. The high pH of *Cucurbita maxima*, and the lower concentrations of all its solutes are noted. Insufficient samples were obtained to allow for nitrogen determinations.

In order to make comparisons between phloem exudate and parenchyma sap from the same plants, similar analyses were carried out for the samples of parenchyma sap collected as described previously. Results of these analyses are included in table VII and show low pH values of parenchyma sap for all species studied with respect to the pH of the phloem exudate. In general the concentrations of calcium, magnesium, potassium, lithium, and total and nitrate nitrogen are about the same in parenchyma sap as in phloem exudate. Whether or not leaf sap and phloem exudate would have been more similar or more divergent if taken at the same time cannot be determined from these data. The amounts of reducing sugars are in excess of the sucrose in all cases while the total sugar concentration is much less than that of the phloem exudate.

TABLE VII

PARENCHYMA SAP ANALYSIS

	Fraxinus americana		*Fraxinus pennsylvanica* var. *lanceolata*		*Robinia pseudo-acacia*	*Platanus occiden-talis*	*Cucur-bita maxima*
	DATE						
	JUNE 5	JUNE 5	JUNE 5	JUNE 5	JUNE 5	JUNE 5	JUNE 5
TREE NO.	2	3	1	2	1	2	
pH	5.63	5.70	5.44	5.80	6.82	4.93	6.11
Ca *mg/cc*	0.72	1.08	1.08	0.72	0.90	1.19	0.54
Mg *mg/cc*	0.38	0.64	0.58	0.32	0.32	0.69	0.12
K *mg/cc*	0.86	1.03	0.93	0.64	1.07	0.74	0.69
Li *mg/cc*	Trace	Trace	Trace	Trace	Trace	Trace	Trace
REDUCING SUGARS *mg/cc*	13.66	12.07	12.01	6.36	3.1	10.26	3.50
SUCROSE *mg/cc*	2.20	1.87	7.18	4.06	1.68	5.45	1.33
TOTAL SUGARS *mg/cc*	15.86	13.94	19.19	10.42	4.78	15.71	4.83
NITRATE NITROGEN *mg/cc*	0.045	0.045	0.087	0.077	0.104	0.098	0.075
TOTAL NITROGEN *mg/cc*	0.070	0.056	0.28	0.112	0.14	0.14	0.84

Qualitative spectroscopic analyses of concentrated solutions of the ash of both phloem exudate and parenchyma sap indicate the presence of barium, boron, copper, manganese, and strontium in varying undetermined amounts in all cases.

Discussion

It has been generally accepted for some time that organic materials are transported through the phloem tissues of plants. The presence of large amounts of organic materials such as sugars, which may reach values as high as 20 per cent. of the exudate, and nitrogenous compounds in the phloem exudate of several species tends to support this idea of transport of organic materials through the phloem. There has not been the same unanimity of agreement with respect to the transport of inorganic materials. The majority of investigators claim that inorganic materials are carried upward primarily through the xylem. CURTIS (**4**) however has presented irrefutable data that inorganic as well as organic materials may be transported through the phloem tissues. The presence in the phloem exudate of such inorganic constituents as calcium, magnesium, potassium, and nitrate nitrogen in amounts appreciably greater than in the parenchyma sap, as well as the presence of small quantities of barium, boron, copper, manganese, and

strontium lends support to the idea of transport of inorganic materials through the phloem tissues. The absence of an element from the phloem would indicate that it was not transported there while the presence of an element in the phloem exudate would offer presumptive but not conclusive evidence of its transport through those tissues.

An interesting observation was made in connection with the flow of the exudate. Collection was begun about August 1st and the flow was good until the first week in October just before the leaves began to change color and fall. The following spring no flow from the phloem tissues could be obtained until about June 15 at which time it was very meager. By July 15 the flow was approximately equal in volume to the flow in late summer of the previous year. The fact that the flow was very slight during the spring and early part of the summer when the cambium is most active lends support to the idea that the exudate was almost wholly limited to the phloem.

It was noted that the total sugar concentration of *Fraxinus americana* decreased as the samples were taken later in August and in September.

Mention has been made of the effect of the proximity of a second cut on the flow from a previous cut.

Summary

1. The pH value of phloem exudate was found to range between 7.05 and 8.10 while that of parenchyma sap ranged between 4.93 and 6.82.

2. Calcium, magnesium, potassium, and lithium were found to be present in the phloem exudate sometime in amounts greater than in the parenchyma sap.

3. The amount of reducing sugar in the phloem exudate is much less than the amount of sucrose except in the case of *Platanus occidentalis*, and the reducing sugar here may have resulted from hydrolysis of sucrose after collection.

4. The total sugar concentration of phloem exudate is much greater than that of the parenchyma sap and may reach 20 per cent. of the exudate.

5. In parenchyma sap the amount of reducing sugar present exceeds the amount of sucrose.

6. Total nitrogen, and nitrate nitrogen concentrations of phloem exudate exceed those of parenchyma sap.

7. Barium, boron, copper, manganese, and strontium are present in both the phloem exudate and parenchyma sap.

8. The presence of appreciable amounts of inorganic constituents in the phloem exudate is presumptive evidence that inorganic as well as organic materials are transported through the phloem.

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CARBOHYDRATE METABOLISM OF *VITIS VINIFERA*: HEMICELLULOSE

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Literature

A review of plant science textbooks reveals the general opinion that hemicellulose often functions as a reserve food. This viewpoint has probably arisen from consideration of the early findings of Sachs (**36**), Reiss (**34**), and Schulze (**37**) that hemicellulose appears to be a major food in certain seeds.

The more recent studies on the composition of hemicellulose have led to other theories as to its physiological function besides those of reserve food and structural reinforcement. O'Dwyer (**31**) has suggested that since hemicellulose is closely related to pectins it serves as an intermediary form in a synthesis of lignin from pectic compounds. The support for this theory is founded on the supposed decrease of pectin during the period of tissue maturation and on the apparently successful attempts to convert pectins to hemicelluloses by mild alkaline hydrolysis (**5**), by hydrolysis with water under pressure (**21**), and by mild oxidation (**28**). The last reaction is presumably analogous to conversion *in situ* in the plant. Although it has been possible to demonstrate the transformation of pectin into hemicellulose, there is no support for the further transformation of hemicellulose into lignin (**32**). Since the structure of lignin has not been established, the theory of the transformation of hemicellulose into lignin must be considered as speculative. Kerr and Bailey (**17**) in their microchemical investigations of the cell wall, moreover, found no reason to believe that pectins disappear with cell maturation. A number of investigators have isolated a small amount of pectin substances from mature wood. The apparent percentage decrease in pectin accompanying maturation appears to be the result of an increasing proportion of other constituents. Difficulties of extraction engendered by mechanical protection or perhaps by a physico-chemical union afforded by the later deposits may possibly have decreased the analytical yield of pectin. Hence positive evidence that the absolute amount of pectin decreases is lacking.

Large variations of any of the constituents, notably that of the labile carbohydrate reserves, cause an inverse change in the percentage of other components. This is illustrated by the hemicellulose content of the fruit of the apple (**13, 24**), the peach (**27**), and the pear (**22**). In each case with maturation a decreasing hemicellulose content was found on a percentage basis which led to the opinion that hemicellulose is utilized as a reserve food in the development of the fruit. Where the hemicellulose content has been expressed on a single fruit basis, however, there is a steady increase in the

absolute amount of hemicellulose in the fruit during growth and ripening, as WIDDOWSON (**42**) has demonstrated for the apple, and SMITH (**38**) for the Kieffer pear. In other words, hemicellulose deposition has only slowed down in respect to the rate of deposition of other substances in the fruit. Working with flowers and young fruits of the apple, HOWLETT'S (**14**) data show no such rapid reversible changes of hemicellulose to sugars as MURNEEK (**24**) found. WIDDOWSON'S data seem to show a slight decrease in the absolute amount of hemicellulose as the fruit becomes overripe, but this might be the result of tissue disintegration which in all probability has no connection with the utilization of hemicellulose as a reserve. BUSTON (**4**), studying the pods of ripening peas and beans, found a decrease in the hemicellulose content on a percentage basis but the data for hemicellulose in grams per hundred pods showed a steady increase.

Sufficient care has not always been taken to formulate an adequate conception of a reserve food. GARDNER, BRADFORD, and HOOKER (**12**) state, "The seasonal variation in the carbohydrate of plants gives evidence of storage." It seems necessary to make the further limitation that fluctuations which do not clearly result from conversion to other forms (such as starch to sugar in early winter) should correlate with conditions of vegetative activity which lead to utilization or storage of reserves. A large percentage utilization under extreme carbohydrate depletion should also be observable. In this latter connection the variability in composition of the hemicellulose extract may raise the question as to whether or not we are dealing with the extraction of a homogeneous fraction or with the simultaneous extraction of several fractions of hemicellulose. Recent studies of hemicellulose by O'DWYER (**30**), NORRIS and PREECE (**29**), and EHRLICH (**11**) have indicated its varying composition—the hydrolysis products yielding hexose and pentose sugars together with a quantity of uronic acid. One such constituent or group of constituents may serve a structural function; the other, perhaps a minor fraction, may serve as a reserve food. If this were the case the utilization of a large proportion of the hemicellulose present would not be observable.

CZAPEK (**7**) and KOSTYTSCHEW (**18**) and others have emphasized the importance of particular hemicellulose constituents as reserves, indicating a less prevalent belief that only certain fractions of the hemicellulose group may function primarily as reserves.

Still another type of finding that has led to an apparently erroneous conclusion is illustrated by the data of NELSON (**25**) and of LEUKEL (**20**) which show small decreases in hemicellulose in alfalfa roots that had undergone repeated top cutting treatments, and of NIGHTINGALE (**26**) which show a significant percentage decrease of hemicellulose in tomato stems grown in darkness for an extended period. These decreases most likely represent the

net effect of a lessened hemicellulose deposit in the newly formed tissue on the percentage of the hemicellulose in the entire plant part utilized. This is confirmed by ALBERT's (1) data on alfalfa which show that new top growth produced in the dark room had a hemicellulose percentage much lower than normal. The difference would have been larger had the hemicellulose content of the normal material been expressed as percentage on a carbohydrate-(starch and sugar) free basis. The evidence seems to indicate that a larger or smaller quantity of hemicellulose may be laid down according to the nutritional conditions within the plant.

A number of investigations show no particularly significant variations in the hemicellulose content. Only a few of these need be mentioned, chiefly to indicate some of the plants that have been studied. DENNY (10) seems to have found no consistent variation in the hemicellulose content of leaves of tobacco, sunflower, cotton, grape, hawthorn, peach, bean, and lilac, using twin-leaf samples collected at night and in the morning. On the average his results indicate a slight gain. JONES and BRADLEE (16) found very small seasonal variations in the maple tree. BENNETT (2) in a seasonal study of the apricot found no reason to believe that hemicellulose functioned as a reserve. WILLIAMS (43) working with the dewberry found that the acid hydrolyzable material showed minor seasonal changes. L. D. Davis (unpublished data), working with bearing and non-bearing trees of the sugar prune, found no significant seasonal variation in hemicellulose although large fluctuations occurred in the starch content (8) of the same material.

MURNEEK (24), LEONARD (19), and CLEMENTS (6) support the theory that hemicellulose serves as a reserve food in the plant. In their investigations, the observed variations of the hemicellulose content were irregular, and furthermore, are not correlated with the vegetative condition of the plant. Their variations certainly do not point to hemicellulose as a reserve, but may have arisen largely from sampling and analytical errors. Arbitrary chemical determinations of hemicellulose may show comparatively large deviations.

Several investigators have employed treatments leading to carbohydrate utilization and have studied the effect on the hemicellulose content. DELEANO (9) divided carefully selected leaves of *Vitis vinifera* along the midrib. One portion served as a check and the other was placed in a light-excluding respiration chamber for varying periods of time from 22 to 288 hours. Under these conditions of decreasing carbohydrate reserves, the percentage of hemicellulose seemed to increase slightly. This increase might be ascribed to continued hemicellulose deposition in the leaf, but it more probably was caused by the effect of the decrease of other constituents on the percentage of hemicellulose. A slight increase of total nitrogen was also shown. If the hemicellulose data are corrected, using the nitrogen increase as a basis, the resultant figures for hemicellulose are remarkably constant. Although

BUSTON (4) found a decrease in the hemicellulose content in grape leaves held in darkness, however, his findings are limited. PROEBSTING (33) in experiments on the apple found that continued defoliation did not decrease the hemicellulose content. The work of ALBERT (1), in which he endeavored to exhaust the reserve from alfalfa roots by growth under conditions of light exclusion, resulted in a consistent increase in the percentage by weight of hemicellulose, doubtless an effect of the carbohydrate utilization on the percentage composition of the residue. It is apparent that hemicellulose was not utilized to any appreciable extent as a reserve under these conditions.

Experimentation

The analytical data, presented in table I, were obtained from representative cross-sections of grape stems (*Vitis vinifera* var. Carignane). The labeling of the sections selected, as indicated in table I, is self-explanatory, except that the term shoot is used to indicate the herbaceous growth of the current season as opposed to the designation cane, used to indicate the mature stems of the preceding season. The data on the annual hemicellulose level were obtained by analysis of composite samples of each of the various sections, obtained by the removal of ten vines at each sampling, at intervals varying from a few days up to one month during the course of the year period. The same sampling procedure was utilized in the severe defoliation treatment, except that only six vines were removed for the composite sample. The defoliation treatment consisted of leaf removal at intervals sufficiently short that the leaves never reached a diameter of more than 2 inches. The defoliation was repeated until the vines were unquestionably in a dying condition. Adjacent vines receiving the same treatment were dead at the time of sampling. The composite samples were dried rapidly in a strong air blast at 55° to 60° C.

The sugars were removed by extraction for 6 hours with 95 per cent. ethyl alcohol. The starch was liquefied by buffered taka-diastase (previously precipitated from water solution by alcohol to remove most of the blank). The residue from the starch extraction was hydrolyzed with 2 per cent. HCl (by weight) for three hours in an enclosed steam bath. The resulting sugars were determined by the MUNSON-WALKER-SHAFFER-HARTMAN method.

It is realized that this analytical procedure includes other polyuronides which are present (chiefly pectic substances) but there is no reason to believe that this affects the conclusions drawn. The data represent averages of the results of duplicate analyses of each sample. All results are expressed in terms of dextrose as percentage of the dry carbohydrate-free residue (starch and sugars) following the example of MASON and MASKELL (23). DENNY (10) found that this procedure gives results compatible with those calculated on the unit organ basis. Large variations in the stored starch have intro-

TABLE I

HEMICELLULOSE CONTENT OF *Vitis vinifera* VINE SECTIONS. PERCENTAGE ON A CARBOHYDRATE-FREE DRY WEIGHT BASIS

DATE	NOV. 6	DEC. 6	JAN. 8	FEB. 10	MAR. 10	MAR. 25	APR. 1	APR. 20	MAY 5	MAY 13	MAY 26	JUNE 7	JULY 6	AUG. 8	SEPT. 6	OCT. 6	NOV. 6	AVERAGE	REPEATEDLY DEFOLIATED VINES
Rootlets	10.4	11.0	10.3	10.9	11.0	11.2	10.5	11.2	10.9	10.5	10.8	11.6	10.1	10.5	11.0	11.2	11.0	10.8	
1 year root wood	13.1	13.4	13.5	13.1	14.1	14.1	14.4	14.3	14.1	13.5	14.8	15.4	14.3	14.3	14.5	14.7	14.5	14.1	14.7
1 year root bark	8.0	8.1	7.7	8.7	8.8	8.0	7.1	7.8	7.7	7.7	8.3	8.4	7.6	7.6	7.8	7.6	7.4	7.9	7.5
2 year root wood	15.6	15.7	16.0	15.6	15.4	15.7	15.8	15.7	16.3	17.1	16.8	16.9	17.1	17.0	15.5	16.4	16.0	16.2	15.6
2 year root bark	10.7	9.9	10.0	10.0	10.1	9.6	9.9	8.4	8.5	10.2	9.8	8.6	8.8	10.3	9.3	8.9	9.1	9.5	7.9
3 year+trunk wood	19.2	19.4	20.2	20.1	19.2	20.3	19.9	20.1	19.6	19.2	20.0	20.1	19.8	19.7	19.1	19.8	19.3	19.7	20.0
2 year trunk wood	21.8	21.9	22.3	22.4	21.7	21.3	22.6	20.6	20.6	21.6	21.5	20.5	21.0	20.2	20.5	20.8	20.4	21.2	20.8
1 year trunk wood	19.9	19.5	19.8	19.9	19.6	19.9	19.4	20.9	20.9	20.7	20.2	20.8	20.4	20.5	20.9	20.5	21.0	20.2	20.5
Trunk bark	10.8	11.8	12.1	11.7	11.8	10.4	11.4	9.8	11.4	10.9	12.5	10.3	11.9	11.1	11.7	12.4	12.1	11.4	12.4
Canes basal wood	21.8	21.0	20.6	20.2	20.7	20.4	21.3	21.1	21.1	22.1	21.6	21.7	21.5	21.7	21.1	20.7	20.0	21.1	20.2
Canes basal bark	14.1	13.8	13.5	14.4	15.1	15.0	13.6	14.0	12.7	13.5	12.7	12.8	13.5	12.5	13.8	14.1	13.8	13.7	14.2
Canes middle wood	19.3	20.0	19.3	19.5	19.2	19.7	19.8	19.8	20.5	19.3	19.4	19.7	20.3	—	—	18.8	19.2	19.6	
Canes middle bark	14.2	14.4	13.6	15.3	15.6	16.1	15.7	13.6	13.3	13.3	12.4	12.2	14.5	—	—	—	13.8	14.1	
Shoot basal wood								7.0*	11.9*	16.1	17.9	16.2	17.0	17.6	18.4	17.7	17.8	17.3	
Shoot basal bark								11.5	12.4	13.0	12.3	12.5	12.0	13.4	14.0	13.7	13.9	12.9	
Shoot middle wood										16.2	18.0	17.2	17.6	18.4	17.6	17.8	18.3	17.6	
Shoot middle bark										12.9	12.3	12.6	11.8	13.5	14.6	14.8	14.1	13.3	
Mature leaves	6.4							6.7	7.1	6.8	6.6	7.0	6.6	6.6	5.7	5.9	—	6.5	

* Not included in the average.

duced apparent but erroneous inverse fluctuations in the data on the hemicellulose content.

Discussion

The most striking feature of the data (table I) is the lack of significant variation between the samples of each series except that for the base of the shoots. In the first sample of this series, taken when shoots were very succulent, the hemicellulose content was less than half that of the usual content (approximately 7 per cent. against the average of later values of 17.3 per cent.) after the tissues had become more mature. At the second sampling, when the base of the shoot was becoming woody, there was still a significantly low hemicellulose content in this section of the vine (12 per cent.). It is interesting to note that these first samples present an inverse curve of the reducing sugar content which decreases rapidly between May 5 and May 26. Starch deposition was relatively very small until approximately a month after the hemicellulose had attained its normal magnitude. At this time the base of the shoot was no longer succulent.

The appearance of a normal hemicellulose content during the period of rapid growth and before starch storage actively begins, points to its function as a structural material. This theory is supported by the lack of significant fluctuation in the hemicellulose content in the other sections. The starch content, which is the principal reserve in this case, fluctuated widely in each part of the vine represented by the samples analyzed. In certain cases (bark samples of canes and trunk) starch was absent during the period of the summer minimum. The non-utilization of hemicellulose in periods when the starch is deficient in certain areas of the vine or at the low summer minimum seems to show that it does not normally serve as a reserve food in the vine.

Although the above results indicate that hemicellulose does not function normally as a reserve food in the vine, it seemed desirable to determine whether or not it *could be utilized* as a reserve of "last resort" as has been proposed by NIGHTINGALE (**26**). In the vines dying because of repeated defoliation treatments, the hemicellulose was found to be present in normal amounts. It is apparent (see table I) that hemicellulose was not utilized as a food reserve even under these conditions of abnormal depletion of carbohydrate reserves. There always remained a detectable amount of starch (with iodine test) in the woody stems. Perhaps this residue was unavailable or was being hydrolyzed very slowly to soluble carbohydrates.

The data for the average hemicellulose values of the various vine cross-sections in table I appear to support the theory of the structural function of hemicellulose. There is a correlation with general observations on comparative thickness of all walls—structural rigidity—of the various tissues with their respective hemicellulose contents. The hemicellulose content was greatest in the xylem of the woody stem, successively decreasing in the

root-wood, bark, and leaves. A number of investigators, including THOMAS (**40**), PROEBSTING (**33**), and SMYTH (**39**), have noted the difference in hemicellulose content between the wood and the bark of the stem. One year MURNEEK (**24**) found less hemicellulose in leaves than in wood but not consistently less in the following year. These differences are presumably caused by the greater amount of hemicellulose in the walls of xylem cells than in those of phloem and cortex cells. The decreased ratio of cortex to xylem in older roots correlates with the increasing hemicellulose content (table I). BENNETT'S (**2**) data show distinctly less hemicellulose in lateral roots than in trunk wood for the apricot.

The mature wood had slightly more hemicellulose than the young shoots. This is an agreement with the results of TOTTINGHAM, ROBERTS, and LEPKOVSKY (**41**) who found less hemicellulose in the new spur growth as compared with that of the old spurs or base wood. Neither of these findings seems to agree with the conclusions of BRADBURY AND ROBERTS (**3**) that acid hydrolysis attacks the young xylem, phloem, and cambium cells rather than the "reserves" occurring as wall thickenings. Probably the increased hemicellulose content of older wood is caused by further hemicellulose deposition. It is reasonable, however, to assume that nutritive or other conditions might reverse this result by influencing the amount of the hemicellulose deposit which is laid down or at least might affect its percentage ratio.

Conclusions

A critical review of the data presented in the literature reveals lack of definite evidence to support the assumption that hemicellulose functions as a reserve food in the vegetative part of plants. On the contrary, a preponderance of evidence indicates that it does not function as a reserve. Definite evidence of its utilization in the seeds of a few plants seems to have been obtained but it appears wise to avoid the reasoning that if hemicellulose is a reserve food in the seed, it will necessarily perform this same function in the plant.

The data of this investigation obtained from *Vitis vinifera* stems fail to show any indication of the metabolic utilization of hemicellulose and support the theory that hemicellulose does not function as a reserve material in the vine even as a reserve of "last resort." The data further indicate that hemicellulose probably functions as a structural material.

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ACCESSORY SALTS IN THE NUTRITION OF EXCISED TOMATO ROOTS

PHILIP R. WHITE

(WITH FOUR FIGURES)

Introduction

In a recently reported study of the effect of vitamin B_1 on the growth of isolated tomato roots (**9**), evidence was obtained which showed that the "accessory salts" regularly included in the medium employed in earlier work (**7, 8**) constituted a growth-limiting factor. Vitamin B_1 proved to be beneficial only in the presence of these salts. That result has since been repeatedly verified. The salt mixture used was modeled after that of TRELEASE and TRELEASE (**3**). It was thought that the ions of its 12 salts might take the place of unidentified but important ash constituents contained in the yeast extract which it was desired to replace. No effort was made to determine the relative importance of the constituents.

With the establishment, however, of a synthetic nutrient capable of maintaining continuous growth of isolated roots, at a fairly satisfactory level, a quantitative examination of all its constituents appeared to be an essential step in attempting further improvement of this nutrient (**9**). The accessory salt mixture was the only portion of the nutrient which had not been subjected to at least a preliminary examination of this sort (**4, 7, 8, 9**). The observations of ROBBINS, WHITE, MCCLARY, and BARTLEY (**2**) also suggested that the ash constituents might be worthy of closer examination.

The present paper records the results of a quantitative study of the effects of each of the components of this accessory salt mixture. The materials and general methods employed were the same as those reported in earlier papers (**7**). The roots had been maintained for 200 or more passages under standard conditions (**6**) before being subjected to experimental treatments.

Experimental results

The accessory salt solution under consideration contained in each liter of complete nutrient the following salts in the amounts shown below:

$Al_2(SO_4)_3$	1.07 mg.	KI	0.15 mg.
NaCl	0.56 "	As_2O_3	0.15 "
$MnSO_4$	0.44 "	$NiCl_2$	0.04 "
H_3BO_3	0.32 "	$CoCl_2$	0.04 "
$ZnSO_4$	0.30 "	LiCl	0.03 "
Na_2SiO_3	0.27 "	$CuSO_4$	0.01 "

Total 3.38 mg.

Since it had previously been found that concentrations of the standard salts optimal for tomato roots were about one-tenth those commonly recommended

for entire plants (**4**), the concentrations of the accessory salts were likewise adjusted to about one-tenth those recommended by TRELEASE and TRELEASE (**3**). The optimal concentration of the entire mixture was then determined experimentally. A series of solutions was prepared containing sugar, standard salts, amino acids, and vitamin B_1 at the standard concentrations (**7, 8, 9**) and the accessory salts at 1, 3, 10, 30, and 50 times the concentrations tabulated above. Twenty cultures were grown at each concentration. Cultures were carried through three consecutive passages, each of one week duration. They were measured daily, the measurements averaged to obtain a weekly index and this recorded as percentage of the control grown in a nutrient containing yeast extract. A "negative control" was also maintained, in which neither yeast extract nor its equivalent (amino acids, vitamin B_1, and accessory salts) was used. The results are shown in figure 1. In the first

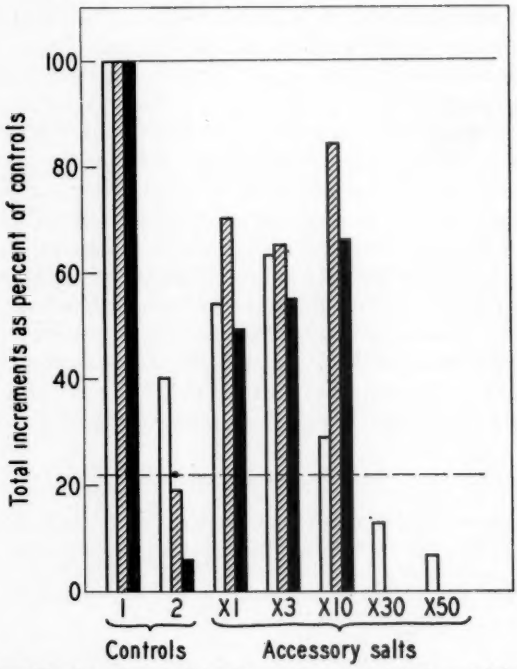

FIG. 1. Growth rates of excised tomato roots in a nutrient containing sugar, standard salts, amino acids, and vitamin B_1, to which various concentrations of an accessory salt mixture were added. Control 1 contained yeast extract in place of amino acids and vitamin. Control 2 was without organic material other than sugar. Open columns represent the first, hachured the second, and blocked-in columns the third passages. The transverse dotted line is at the level of the average growth rate (3 passages) without accessory material. Any growth rate below this level indicates a depressant effect.

passage growth at concentrations 10, 30, and 50 times the standard was less than in the negative control, that is, these solutions had a depressant effect. The last two concentrations were, therefore, abandoned. Growth was best at a concentration 3 times the standard in the first passage, but there was a gradual recovery at 10 times the standard so that in the second and third passages this nutrient gave superior results. It thus appears that tomato roots may become adapted to growth in salt concentrations higher than those which are at first optimal. For subsequent studies a concentration between these two values, 5 times the original standard, was chosen. Such a nutrient contained 17 mg. of accessory salts per liter.

The importance of each salt was then studied in an "all or none" series. Sixteen nutrients were prepared. Each, with the exception of the negative control, contained the standard salts, sugar, amino acids, and vitamin B_1. Four controls were used, one containing yeast extract, one without accessory material but with standard salts and sugar, one containing all 12 of the accessory salts, and one from which all these salts were omitted. In each of the remaining solutions one of the 12 accessory salts was omitted. Twenty cultures were prepared in each nutrient and were maintained through 3 passages. The results are shown in table I.

Of the 12 salts studied, only 2, aluminum sulphate and potassium iodide,

TABLE I

GROWTH INDICES OF TOMATO ROOTS CULTIVATED IN NUTRIENTS EACH LACKING A SINGLE ONE OF THE ACCESSORY SALTS, EXPRESSED AS PERCENTAGE OF THE GROWTH IN A CONTROL NUTRIENT CONTAINING YEAST EXTRACT

SOLUTION	PASSAGE			AVERAGE
	1	2	3	
With yeast 1	100	100	100	100
Without yeast 2	34	43	17	28
All accessory salts omitted 3	68	50	49	56
All accessory salts included 4	100	79	95	91
Minus Al	108	106	110	*108*
" Li	88	93	110	97
" Zn	102	81	95	93
" Mn	92	74	102	89
" Ni	98	65	97	87
" Cu	91	84	85	87
" Si	82	85	96	87
" Na	97	86	81	87
" Co	90	79	85	85
" As	102	74	64	80
" B	73	74	80	76
" I	64	56	60	*64*

gave sufficiently clear-cut results to be of certain significance. Aluminum sulphate (1 p.p.m.) was clearly detrimental. Potassium iodide (0.15 p.p.m.) was equally clearly beneficial. All other salts were of doubtful value. In view of the uncertainty of these results, an arbitrarily chosen combination was selected for further study. For this, 6 salts only were chosen: potassium iodide because the preliminary study had suggested its importance; manganese and zinc sulphates and boric acid because, in spite of their apparent unimportance in this first study, the literature of plant nutrition contains so many rather clear-cut demonstrations of their desirability (the first two are likewise frequently found as impurities in salts of other metals and may have been supplied in sufficient amounts in the 11 salts present in the "minus Mn" and "minus Zn" series); sodium silicate because MAST and PACE (1) have suggested its importance for certain organic catalyses; and cobalt chloride because of the recent interest in the possible rôle of cobalt in animal and plant nutrition. WINKLER years ago (10) pointed out the possible growth-stimulating effects of traces of cobalt. Since it was desired to test 4 concentrations of each salt and since the maximum number of cultures which could be maintained at one time under uniform conditions in the laboratory available was 320, no more than 4 salts could be compared simultaneously. Salts of iodine, boron, manganese, and silicon were studied first. Nutrients were made up containing 5 of the 6 salts chosen, at standard concentrations, while the sixth salt was introduced at 0.0, 0.1, 1.0, and 10.0 times the concentrations employed in the earlier series. The experiment was conducted as before.

The results, shown in figure 2, indicate clearly that iodine was essential

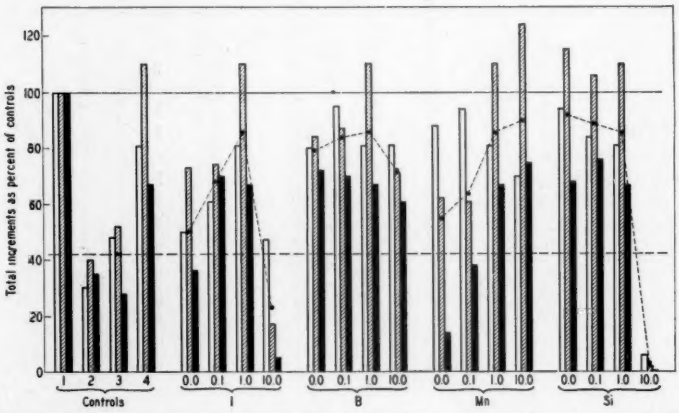

FIG. 2. Legend as for fig. 1. Control 3 is with amino acids and vitamin B_1 but without accessory salts. Control 4 is the same as 3 but with I, B, Mn, Si, Zn, and Co added (see text). The transverse dotted line is placed at the level of the mean growth rate for control 3, without accessory salts. Solid dots are in each case placed at the average (3-week) growth rate for the nutrient indicated.

for satisfactory growth of isolated tomato roots. A concentration of about 0.75 p.p.m. of KI was about optimal. Manganese was likewise highly desirable, with an optimal concentration at about 4.40 p.p.m. of $MnSO_4$. Enough Mn seems to have been present in the "minus Mn" nutrient for fairly satisfactory growth. Boron was possibly beneficial with an optimum at about 1.60 p.p.m. of H_3BO_3. Some boron was, of course, supplied to the cultures by solution of the glass (Pyrex) in which they were grown. A clear-cut deficiency was thus not to be expected in this case. Silicon proved to be markedly detrimental, the growth rates showing a regular diminution with increasing concentration of Na_2SiO_3. In two solutions, that with increased Mn concentration and that from which silicon was omitted, the growth indices were greater than when all 6 salts were present at the concentrations used before, and were superior to any results obtained in earlier nutrients containing all 12 salts.

Using these results as a basis, a series was then prepared in which Si was omitted, the concentrations of I and B were maintained at the basic values, the concentration of Mn was increased by 10, and the concentrations of Zn and Co were varied as was previously done with the other ions. The results, shown in figure 3, indicate that zinc was probably beneficial, with an optimum at about

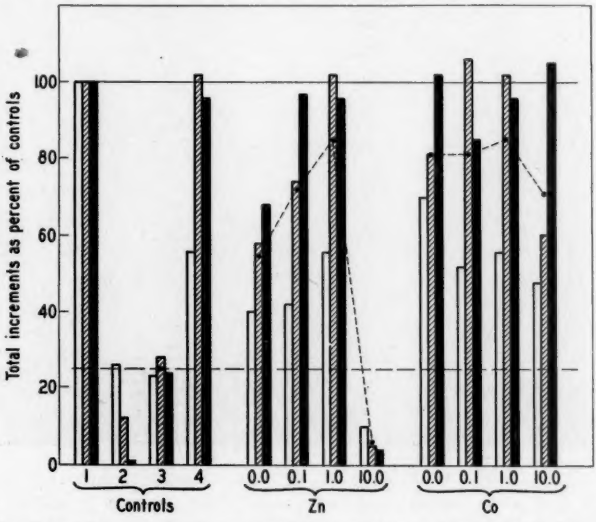

FIG. 3. Legend as for fig. 2, except that control 4 contains only I, Mn, B, Zn, and Co, without Si.

1.5 p.p.m. of $ZnSO_4$, while cobalt, at the concentrations tested, was without significant effect.

It thus appears that nutrients containing only four accessory salts, $MnSO_4$, $ZnSO_4$, H_3BO_3, and KI, are capable of maintaining growth of isolated tomato roots through at least 3 passages without apparent deterioration or diminu-

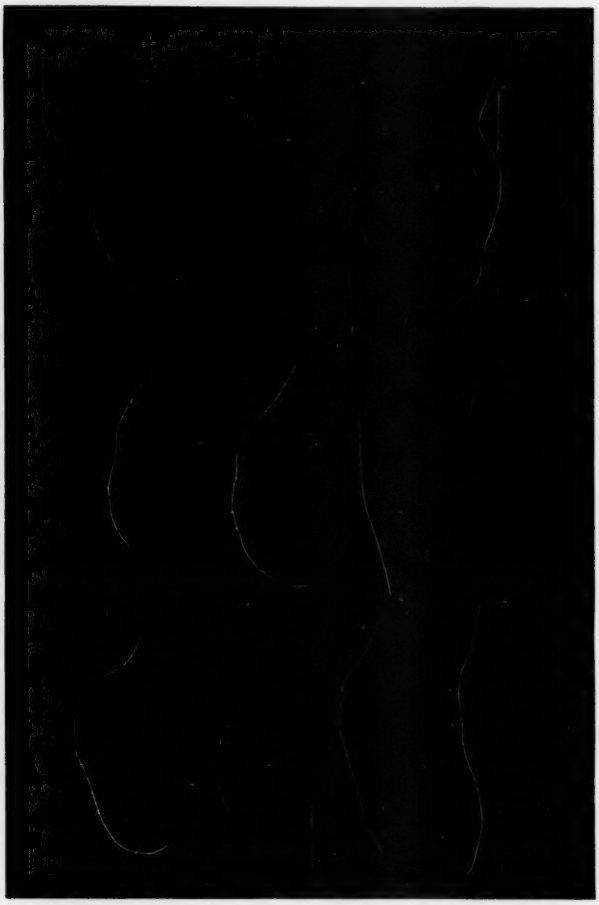

FIG. 4. Roots grown in the following solutions (reading from left to right): Top row, control with yeast extract; negative control without accessory material; nutrient containing standard salts, sugar, amino acids, and vitamin B_1 but with no accessory salts; same as the last but with Mn, B, I, Zn, and Co added. Middle row, nutrient the same as that in the upper right hand corner except that concentrations of Zn, 0.0, 0.1, 1.0, and 10.0 times the standard, were used. Bottom row, like the middle row but with concentrations of Co varied, instead of Zn. All pictures taken at the end of 3 passages. $\times 0.5$. (Photograph by J. A. CARLILE.)

tion in growth rate and at a level almost equal to that in a yeast extract medium. Addition of any of the other 8 salts originally included in the accessory salt mixture did not improve the result. As is evident from figure 4 (upper right hand corner), the condition of such roots at the end of 3 passages was excellent and the contrast between them and roots grown in a nutrient lacking these 4 salts (fig. 4, second from the right, top row) is striking. The nutrient is little if at all inferior to the control and is superior to any synthetic nutrient heretofore developed.

Discussion

The best synthetic nutrient presented in previous work (9) contained sucrose, 18 salts, 9 amino acids, and vitamin B_1. It was capable of maintaining continuous growth at a level somewhat inferior to that in a yeast extract medium. In discussing that nutrient, it was pointed out that its deficiency might be due to improper balance of the constituents already there, in which case a quantitative study might lead to correction of the deficiency, or it might be due to the absence of some desirable but not absolutely essential constituents, in which case a quantitative study would not be adequate. The present study has shown clearly that the former is, at least in large measure, the case; that is to say, the fault has been largely one of improper balance and of a *too complicated* medium. By simplifying the nutrient through the omission of 8 salts and by modifying the concentration of manganese, the results have been greatly improved and brought up to almost, if not quite, the level of the control containing yeast. The optimal synthetic nutrient developed to date then contains the following ingredients in each liter of solution:

$Ca(NO_3)_2$	70.00 mg.	dl-phenylalanine	1.50 mg.
KNO_3	80.00 "	dl-lysine	1.50 "
KCl	65.00 "	dl-valine	0.15 "
KH_2PO_4	12.55 "	dl-serine	0.05 "
KI	0.75 "	dl-isoleucine	0.0015 mg.
$MnSO_4$	4.40 "	d-glutamic acid	5.00 mg.
$Fe_2(SO_4)_3$	2.50 "	l-histidine	1.50 "
$ZnSO_4$	1.50 "	l-proline	0.50 "
H_3BO_3	1.60 "	l-leucine	0.015 mg.

Vitamin B_1—1 mg. Sucrose—20 gm.

It seems possible that a re-examination of the amino acid mixture, carried out in a way similar to that just described for the accessory salt mixture, may result in still further simplification of the nutrient, with concomitant improvement.

Summary

Of the 12 accessory salts included in the formula of TRELEASE and TRELEASE, only four, $MnSO_4$, $ZnSO_4$, H_3BO_3, and KI, appear to be essential for

growth of isolated tomato roots under the conditions of the experiments here reported. Other salts may have been introduced as impurities but played no recognizable rôle in the growth of the roots in question. When the four salts named above are added to a nutrient containing the standard salts of Pfeffer's solution, iron, sucrose, amino acids, and vitamin B_1, in proper proportions, the resulting nutrient is almost, if not quite, equal to a yeast extract medium as a source of materials essential for growth of these roots.

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INFLUENCE OF FOLIAGE SPRAYS ON DROUGHT RESISTANCE OF CONIFERS

HARDY L. SHIRLEY, AND LLOYD J. MEULI

(WITH ONE FIGURE)

Introduction

Periodic drought is one of the most serious hazards to young conifer plantations in several regions of this country. In the Lake States region, for example, droughts have occurred in four years of the past seven. Biologists have suggested that repellent substances sprayed on leaves of conifers to prevent rodent damage might, in addition, have a beneficial effect on survival during droughts. It seemed desirable to make a careful test of a few sprays to determine if they were of any real use in increasing resistance to drought. The present paper describes experiments designed to test the protective value of various sprays that have been suggested as useful agents in lowering transpiration.

Review of literature

EMERSON and HILDRETH (**2**) treated ponderosa pine with four different spray materials, all of which caused a pronounced decrease in the rate of transpiration. Corn oil, the most efficient, reduced transpiration from 20.7 to 2.4 gm. daily. These sprays appeared to have no ill effects on the trees and seemed to promote increased survival of seedlings planted in the field.

MALIN (**6**) showed that the rate of transpiration and the osmotic pressure increased rapidly after the natural waxy coating was removed from the needles of conifers. MITTMEYER (**8**) found that the cuticular transpiration is very important in many plants of the xerophytic type, while for mesophytes a much larger percentage of transpiration occurs through the stomata. FÜSSER (**4**) showed that coating leaves with vaseline and other materials designed to reduce transpiration tended to upset their water relations to such an extent that for some leaves the transpiration rate with one surface coated with vaseline was as rapid as from the uncoated leaf. ARTHUR and STEWART (**1**) reported that coating leaves of tobacco plants with vaseline did not reduce transpiration to any appreciable extent when they were exposed under conditions of high light intensity and high temperature.

The effects of sprays on the water balance of plants are reflected in changes in their rate of photosynthesis. HOFFMAN (**5**) reported that lime-sulphur sprays decreased the rate of photosynthesis in apple leaves as much as 38 per cent. Summer oil sprays also caused a marked decrease at first, but after a week the leaves regained their initial rate of assimilation. SCHROEDER (**10**) found that the carbon dioxide absorption of apple leaves

was materially reduced after applications of mineral or vegetable oil sprays. WILSON and RUNNELS (**14**) report increases in transpiration of *Coleus* plants sprayed with copper-containing mixtures varying from 42 per cent. for colloidal copper up to 1000 per cent. for Bordeaux mixture. In an earlier report (**13**) these same investigators found that Bordeaux mixture checked growth, decreased yield, and increased transpiration of tomatoes, while an oil spray apparently had no unfavorable influence on these plants. ROHRBAUGH (**9**) found that heavy oil sprays accumulated in the leaves and twigs of citrus fruit trees where they persisted for as long as 6 months, apparently causing only slight injury to the trees. MCKAY (**7**), on the other hand, found that paraffin oils injured considerably the young shoots of apple trees. The evidence from the literature on the subject of protective sprays is somewhat conflicting, and might lead one to question whether foliage sprays provide sufficient protection against excessive transpiration to offset their unfavorable effects.

Experimental methods and results

Three conifers, Norway pine, *Pinus resinosa* Solander, white pine, *Pinus strobus* L., and white spruce, *Picea glauca* (Moench) Voss., were used in these tests.

Sprays, furnished by the Central Great Plains Horticulture Field Station at Cheyenne, Wyoming, were made up as follows:[1]

No. 1:

Beeswax	10 parts (weight)
Fish oil soap	1 part
Water	50 parts

Dissolve the soap in water by heating to 95° C.; melt the beeswax and heat to 70° C.; add beeswax *slowly* to heated soap solution while stirring vigorously.

No. 2:

Parawax	5 parts (weight)
Beeswax	5 parts
Fish oil soap	1 part
Water	40 parts

Prepare the same as no. 1.

No. 3:

Goodrich rubber solution	1 pound
Fish oil soap	2 ounces
Water	2 ounces

Dissolve the soap in water and cool to room temperature. Add the rubber solution to the soap solution while stirring vigorously.

No. 4:

Hydrowax liquid N full strength.[2]

[1] The writers are indebted to J. L. EMERSON, formerly of the Central Great Plains

Laboratory tests

Laboratory tests of drought resistance were made in the 1933 drought machine (**11**). Two-year-old white spruce seedlings were potted in tin cans and allowed a week or more to become established. The materials were sprayed on the leaves with a hand sprayer, just before placing the plants in the testing machine. The soil in all plant containers was maintained at the same moisture content by daily additions of water through a glass pipette in the waxy seal. The plants remained in the machine until dead. An untreated control was tested with each lot of sprayed plants.

A measure of the reduction in transpiration might be considered a justifiable estimate of spray values during periods of drought. Consequently records of daily water loss from each plant were kept for the three laboratory tests as shown in figure 1. It will be noted that the more effective sprays

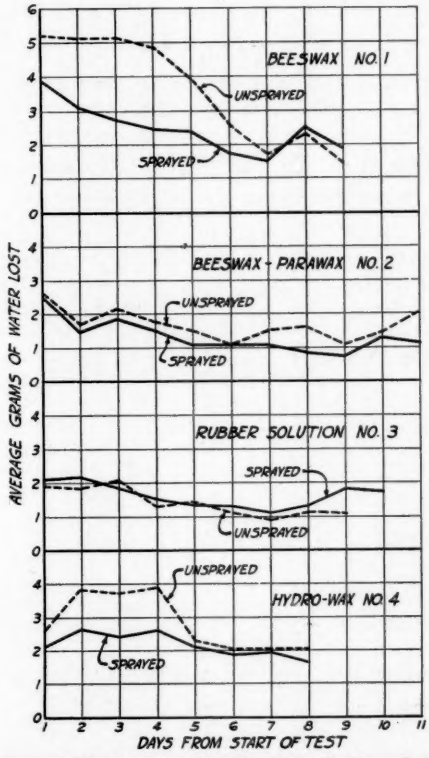

Fig. 1. Effect of foliage sprays in daily transpiration of white spruce.

Horticulture Field Station at Cheyenne, Wyoming, for the sprays used in these tests.

[2] Purchased from Glyco Products Co., Brooklyn, New York.

TABLE I

EFFECT OF SPRAYS ON DROUGHT RESISTANCE OF WHITE SPRUCE. AVERAGE OF 3 LABORATORY TESTS

SPRAY USED	TOTAL NUMBER OF PLANTS SPRAYED AND UNSPRAYED	AVERAGE DAYS SURVIVED IN MACHINE	
		SPRAYED	CONTROL
(1) Beeswax	86	8.34	8.56
(2) Beeswax-parawax	86	9.10	8.93
(3) Rubber solution	81	8.60	8.84
(4) Hydrowax	85	8.63	9.50

caused an important reduction in transpiration during the first four days, but thereafter, as the available soil moisture was reduced, unsprayed and sprayed plants lost water at approximately the same rate.

TABLE II

ANALYSIS OF VARIANCE OF DAYS SURVIVAL IN DROUGHT MACHINE BY SPRAYED AND UNSPRAYED SPRUCE SEEDLINGS

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	OBSERVED F
Spray 1			
	Total 85	9.15	
Between treatments	1	6.15	1.01
Between tests	2	137.29	22.64*
Remainder	82	6.06	
Spray 2			
	Total 85	7.98	
Between treatments	1	.94	.27
Between tests	2	199.13	58.26*
Remainder	82	3.42	
Spray 3			
	Total 80	11.00	
Between treatments	1	5.31	1.62
Between tests	2	310.97	94.84*
Remainder	77	3.28	
Spray 4			
	Total 84	18.73	
Between treatments	1	32.80	6.17†
Between tests	2	555.11	104.43*
Remainder	81	5.32	

* Indicates significance beyond 0.01 level.

† Indicates significance between 0.05 and 0.01 levels.

TABLE III

EFFECT OF FOLIAGE SPRAYS ON FIRST-YEAR MORTALITY OF PLANTED CONIFERS

SPRAY TREATMENT	AVERAGE MORTALITY						
	PIKE BAY EXPERIMENTAL FOREST*			CUTFOOT EXPERIMENTAL FOREST*		WEIGHTED MEAN ALL SPECIES	INCREASE OVER UNTREATED†
	LOT 1 NORWAY PINE 22 GROUPS	LOT 2 WHITE PINE 5 GROUPS	LOT 3 WHITE SPRUCE 18 GROUPS	LOT 4 NORWAY PINE 12 GROUPS	LOT 5 WHITE SPRUCE 8 GROUPS		
	%	%	%	%	%	%	%
Untreated	8.21	0.00	8.05	11.22	14.30	8.84	.....
(1) Beeswax	10.50	3.09	7.12	12.64	14.70	9.90	1.06
(2) Beeswax-parawax	8.24	1.75	11.80	12.35	19.30	11.03	2.19
(3) Rubber solution	9.02	2.19	12.80	12.89	26.08	12.39	**3.55**
(4) Hydrowax	9.22	4.63	12.70	14.28	22.11	12.37	**3.53**

* On the Chippewa National Forest, Minnesota.

† The standard error of these differences is 1.375. Values of 2.75 or greater are considered to be significant and are designated by bold-faced figures.

Data from all tests were grouped together (table I) and treated by FISCHER'S (3) analysis of variance method.[3]

The results are given in table II. It will be noted that differences between tests are highly significant. This was due very likely to the different stages of vegetative development at the time of testing. In only one case is there a significant difference between treated and control plants. Hydrowax liquid N caused a reduction in drought resistance and at the same time caused a considerable decrease in transpiration. Presumably its lowering of drought resistance may be attributed to effects other than those effecting the transpiration rate. All sprays caused the needle tips to turn brown.

During 1937 a new paraffin emulsion appeared, designed to protect plants from desiccation.[4] A sample of this material obtained from the Dow Chemical Company, Midland, Michigan, was diluted with water 1 to 3½ and sprayed on 2-year-old jack pine. A machine test of drought resistance, involving 45 treated and 45 control plants, was made in the usual manner. Both treated and control plants lived an average of 4.9 days.

FIELD TESTS OF DROUGHT RESISTANCE

Field tests were made in two different areas to determine the influence of sprays on field survival. These field test areas, about thirty miles apart, were located in the Chippewa National Forest. During the test period in 1934, precipitation at Cass Lake, Minnesota, was about normal in June, 1.34 inches below normal in July, and 1.87 inches below normal in August. The drought was not quite as severe as those which occurred in other years of the past seven.

A total of 49,000 plants consisting of the three species, Norway pine, white pine, and white spruce, were used in the tests. The plants were sprayed in bundles before planting in the field. At the end of the first growing season 50 per cent. of the planted trees were examined to determine the percentage of mortality. The results of these counts are given in table III. In general, spraying resulted in increased mortality. The only exception was white spruce which, when sprayed with beeswax, had a lower mortality

TABLE IV

ANALYSIS OF VARIANCE OF FIELD MORTALITY OF SPRAYED AND UNSPRAYED CONIFERS

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	OBSERVED F
	Total 324		
Within lots (error)	300	61.57	
Between lots	4	1323.46	21.50*
Between treatments	4	154.14	2.50†
Interaction, lots × treatments	16	55.79	0.91

* Indicates significance at 0.01 level (12).

† Indicates significance at 0.05 level (12).

[3] Assistance with statistical treatment was furnished by R. H. BLYTHE, JR.

[4] MILLER, E. J., NEILSON, J. A., and BANDEMER, S. L. Wax emulsions for spraying nursery stock and other plant materials. Michigan Agr. Exp. Sta. Tech. Bull. 282. 1937.

than unsprayed plants. This advantage occurred only on the Pike Bay Experimental Forest. These data were treated by FISHER'S (3) analysis of variance method as shown in table IV. Differences between lots and between treatments were both significant. The standard error for the mean of all species and sites was computed to determine which particular treatments differed significantly from the untreated. The increase in average mortality over that of untreated plants is also shown in table III.

In field tests, plants treated with both rubber solution and Hydrowax had significantly higher mortality than untreated plants. These sprays must be considered distinctly harmful under the conditions used. The other two sprays were not beneficial.

Discussion of results

Neither in the laboratory tests nor in field survival did sprayed plants exhibit any significant advantage over untreated ones. In the laboratory tests the plants were subjected to severe atmospheric and soil drought. In field tests the drought was only moderate. In view of the rather extensive trial given, it appears logical to assume that none of the four sprays afforded any real protection against drought to the species used. In this region, at least, their effect, if any, was detrimental. Whatever benefit that may have resulted in reducing transpiration of the sprayed plants was evidently offset by disadvantages due to other causes.

Summary

1. Norway pine, white pine, and white spruce were treated with four foliage sprays to determine their efficacy in improving resistance to drought as measured by laboratory and field tests.

2. None of the sprays studied improved resistance to drought in these conifers.

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FORMATION OF NITRATE IN DETACHED GREEN LEAVES OF SWISS CHARD AND TOMATO

MARY C. MCKEE AND DOROTHY E. LOBB

(WITH TWO FIGURES)

Introduction

In 1933, VICKERY, PUCHER, WAKEMAN, and LEAVENWORTH (**6**) reported the formation of nitrate in tobacco leaves subjected, in the absence of light, to either air drying or distilled water culture. The present investigation seeks to extend this unprecedented (**1**) observation to leaves other than those of the tobacco; for this purpose random choice was made of Swiss chard (*Beta vulgaris* var. *cicla*) and tomato *Lycopersicum esculentum* Mill). The treatment of the leaves followed as closely as possible that given the tobacco; the results so obtained indicate that the tobacco is not unique in showing nitrate formation and that the phenomenon differs in magnitude depending upon the nature of the leaf and its condition.

Procedure

SWISS CHARD

PREPARATION OF DRIED LEAF TISSUE.—Two series of investigations were carried out on Swiss chard (var. Lucullus) at the end of three months' growth. The two crops were grown from the same seed, at different seasons of the year, in different locations, and under different nutritive conditions.

Lot A was planted in July, 1936, in an open field and received little cultivation. The leaves were harvested in October. Lot B was planted January 16, 1937, in the college greenhouse. Once a week from the second week after planting until ten days before picking, April 5, the twenty-four plants received a pint of nutrient solution of the following composition: calcium nitrate, 4 gm.; potassium nitrate, 1 gm.; magnesium sulphate, 1 gm.; potassium dihydrogen phosphate, 1 gm.; potassium chloride, 0.5 gm.; water, 1 liter.

In each case the leaves were harvested about eight o'clock in the morning. They were practically free from surface moisture and were brushed free from soil. All except very old and very young, green, healthy leaves were stripped from the plants and, therefore, the samples were of varied sizes and stages of development. The petioles were removed unless the leaves were to be water cultured, the leaves were divided into groups of approximately 100 gm. each, washed in tap water, blotted dry, and treated as follows:

Controls.—Groups A–1 and B–1 were shredded, piled lightly on pyrex plates and dried in an electric oven for five to six hours at 75° to 100° C. Frequent turning of the leaves aided in promoting uniform drying. At the end of this period the leaves were allowed to cool in the air, weighed, ground in a Nixtamal mill or by hand in a glass mortar, and bottled for later determination of nitrate and total solids.

Water culture in the dark.—Leaves of groups A–2, A–3, A–4, B–2, B–3, B–4 were placed in liter beakers with the petioles dipping in distilled water and kept in a dark room at approximately 24° C. for periods of about 48, 72, and 96 hours. Fresh water was supplied and the stems scraped each twenty-four hours. The leaves were turgid and there was no visible evidence of bacterial action. At the end of each culture period the petioles were removed and the leaves oven-dried, weighed, and ground. The weight was corrected for the removal of the petioles.

Air drying in the dark.—Leaf groups A–5, A–6, A–7, B–5, B–6, B–7 were suspended by cords passing through the mid rib of each leaf, in a well ventilated dark room where they dried slowly at 20° to 22° C. for three-, six-, and nine-day periods respectively. Following the curing process, each lot was dried in the oven for three to four hours. This left the leaves a bright green color in contrast to the controls and water cultured leaves which were darkened by oven drying. The dried leaves were air cooled, weighed, ground, and preserved in glass stoppered bottles.

ANALYTICAL PROCEDURE.—Determination of total solids in (a) fresh leaf and (b) dried leaf tissue. (a) Two fresh leaves were shredded and weighed quantitatively in no. 4 porcelain crucibles, oven heated with the controls, transferred from the oven to a vacuum desiccator and dried to constant weight over sulphuric acid (sp. gr. 1.84). The percentage of total solids in fresh leaf as obtained in this way was checked by following the change in weight upon drying of the different batches of leaves.

(b) Duplicate samples (one to two gm. each) of the bottled oven dried leaf material were dried to constant weight *in vacuo* over sulphuric acid (sp. gr. 1.84).

Determination of nitrate-nitrogen in the oven dried leaf.—Following the technique of PUCHER, VICKERY, and WAKEMAN (3), one- to two-gram samples of the oven dried leaf powder were brought to a pH just less than 1 (quinhydrone method) by addition of 4 N sulphuric acid, and the nitric acid so formed was removed by extraction with alcohol- and peroxide-free ether in an underwriter's extraction apparatus. Complete removal of the nitrate ion was tested by use of diphenylamine. The ether extract was diluted with water, neutralized with 0.5 N sodium hydroxide, the ether removed by evaporation, and the aqueous solution made up to 100 ml. Aliquots of 5 or 10 milliliters were reduced with iron powder and sulphuric acid and the ammonia determined by distillation from an alkaline solution into hydrochloric acid and subsequent Nesslerization. A Duboscq colorimeter was used and corrections were made for the nitrogen content of the iron powder and the ammonia content of the leaf ether extract.

TOMATO

Tomato seed (var. Margrove) was planted, April 4, 1936, in the college greenhouse; the young plants were removed to an outdoor garden on May

18. The plants were well fertilized with nutrient solution (described under Swiss chard) and sheep manure, and they grew vigorously. Two lots of clean, healthy, green leaves of varied sizes and ages were harvested, one on the morning of July twenty-second, the other on the afternoon of August eighth. They were treated in a similar manner and subjected to the same analyses as the chard leaves.

Results

The experimental data are summarized in tables I and II. A comparative study of the results obtained with tobacco (VICKERY *et al.*), Swiss chard, and tomato leaves may be made by means of figures 1 and 2.

Discussion

In the analysis of green leaves two difficulties are always met, the procuring of a uniform sample of the fresh leaf and the preparation of representative material for direct analysis. The leaves used in this work were of varied sizes and stages of development and the attempt to surmount the first difficulty lay solely in the use of a large number of leaves well mixed. In table II, under A–1, are recorded the analyses of three separate groups of unselected leaves all subjected to the same experimental procedure. The

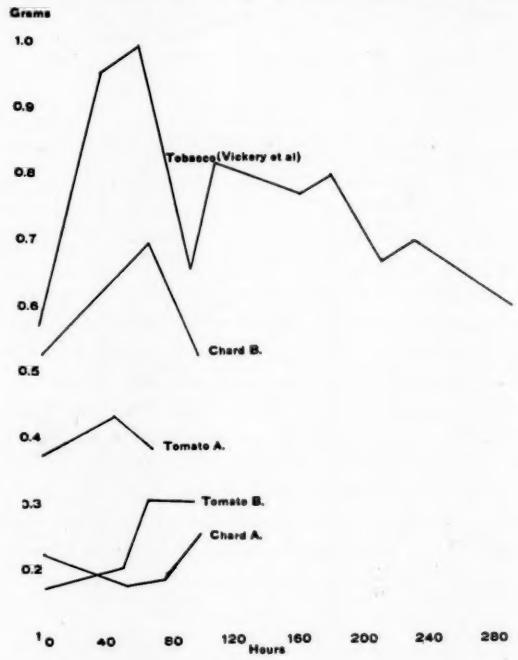

FIG. 1

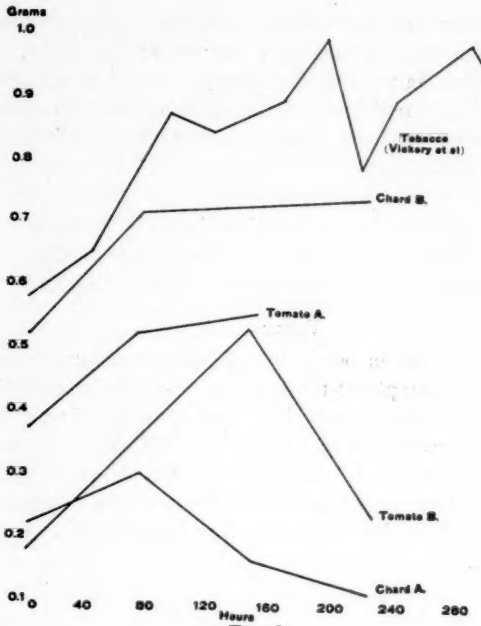

FIG. 2

TABLE I

NITRATE CONTENT OF SWISS CHARD LEAVES

SAMPLE	NITRATE-N IN ONE GRAM OF OVEN DRIED LEAF	TOTAL SOLIDS IN 100 GRAMS OF OVEN DRIED LEAF	NITRATE-N IN ONE GRAM OF TOTAL SOLIDS	TOTAL SOLIDS IN 100 GRAMS OF FRESH LEAF	NITRATE-N IN ONE GRAM OF FRESH LEAF
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
A					
Control, A–1	1.92	94.8	2.03	11.3	0.229
Water cultured					
48 hr., A–2	1.54	94.2	1.63		0.184
70 hr., A–3	1.58	94.6	1.63		0.189
92 hr., A–4	2.11	95.2	2.22		0.250
Air dried					
72 hr., A–5	2.54	94.2	2.69		0.305
144 hr., A–6	1.48	93.6	1.56		0.176
216 hr., A–7	1.04	94.2	1.11		0.125
B					
Control, B–1	7.24	95.5	7.50	7.06	0.530
Water cultured					
48 hr., B–2	8.92	97.7	9.14		0.645
72 hr., B–3	9.70	97.5	9.95		0.703
96 hr., B–4	7.54	98.3	7.67		0.541
Air dried					
72 hr., B–5	9.80	97.1	10.1		0.712
144 hr., B–6	9.71	95.4	10.2		0.719
216 hr., B–7	9.87	94.5	10.5		0.737

TABLE II

NITRATE CONTENT OF TOMATO LEAVES

SAMPLE	NITRATE-N IN ONE GRAM OF OVEN DRIED LEAF	TOTAL SOLIDS IN 100 GRAMS OF OVEN DRIED LEAF	NITRATE-N IN ONE GRAM OF TOTAL SOLIDS	TOTAL SOLIDS IN 100 GRAMS OF FRESH LEAF	NITRATE-N IN ONE GRAM OF FRESH LEAF
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
A					
Control, A–1					
a.	3.35	91.5	3.66	10.52	0.385
b.	3.31	94.1	3.52	10.54	0.371
c.	3.05	88.9	3.43	11.28	0.387
				10.8 av.	
Water cultured					
43 hr., A–2	3.88	94.8	4.09	10.8	0.442
64 hr., A–3	3.54	95.6	3.70		0.400
Air dried					
72 hr., A–5	4.56	93.7	4.87		0.526
144 hr., A–6	4.99	95.5	5.23		0.564
B					
Control, B–1	1.51	91.7	1.64	11.8	0.195
Water cultured					
45 hr., B–2	1.54	89.5	1.72		0.203
66 hr., B–3	2.37	90.0	2.64		0.311
90 hr., B–4	2.37	89.7	2.64		0.311
Air dried					
66 hr., B–5	2.51	91.0	2.76		0.326
138 hr., B–6	4.15	91.1	4.56		0.538
220 hr., B–7	1.97	93.8	2.10		0.248

results show that the differences in values for nitrate and total solid content are well within the range of experimental error and the conclusion that a mixture of many leaves yields fairly uniform samples seems justified.

Many observations (**2, 4, 5, 7, 8,** etc.) confirm the selective storage of nitrates in different structural parts of a plant. It is not possible to insure a similar weight ratio of blade, rib, and petiole in all groups of leaves. Thorough mixing of the ground dried material from a large number of leaves was the method used in meeting this second source of error.

While it is probable that no nitrate was lost in drying at the temperature used, the same assumption cannot be made in regard to volatile oils and, perhaps, to chemically combined water. It may well be that the weight of the total solids varies depending upon whether the leaves are, or are not, air dried before heating. To test this, two one-leaf samples of Swiss chard (lot B) were weighed in duplicate; one was oven heated immediately for five hours, then dried to constant weight in a vacuum desiccator over concentrated sulphuric acid; the other was air dried for three days before it was heated and dried *in vacuo*. The values for the weight of total solids in 100 grams of fresh leaf, as obtained in these two ways, were 7.06 and 7.36 grams. No increased loss of weight had resulted from the exposure of the chard leaves

to the air for three days before heating. A similar check was not made with tomato leaves.

Summary

Swiss chard and tomato leaves when subjected to air drying at room temperature in a well ventilated place showed, for a limited time, an increase in nitrate content. The change was more pronounced in the tomato than in the chard and varied in two lots of leaves grown from the same seed but under different conditions.

Both Swiss chard and tomato leaves when cultured in distilled water, in absence of light, gave evidence of slight nitrate formation. Again the increase in nitrate varied with the nature and condition of the leaf.

These observations are in agreement with that of VICKERY *et al.* who, in 1933, reported an increase in the nitrate content of tobacco leaves during the early stages of curing and water culture.

The writers wish to acknowledge their indebtedness to Dr. H. B. VICKERY and Dr. G. W. PUCHER for advice in carrying out this investigation.

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FORMATION OF CAROTENOIDS AND CHLOROPHYLLS IN ETIOLATED BARLEY SEEDLINGS EXPOSED TO RED LIGHT

HAROLD H. STRAIN

(WITH ONE FIGURE)

Introduction

For the investigation of many of the problems pertaining to the metabolism and function of the leaf pigments, it would be desirable to vary the relative proportions of the several leaf pigments in any given plant through changes in the environment. Average pigment determinations reported by RUDOLPH (1) have already indicated that red light, which promotes the formation of chlorophyll, does not stimulate the formation of the yellow pigments in etiolated bean leaves, but the individual determinations were subject to great variation. Moreover, etiolated bean leaves contain relatively large quantities of carotene and xanthophylls in nearly the same proportions found in green leaves, so that, for demonstration of the effect of red light on the formation of carotenoids in these seedlings, extremely precise determinations of the pigments were required.

Investigations in this laboratory have shown that etiolated barley leaves, in contrast with the etiolated bean seedlings, contain very small quantities of carotene relative to the xanthophylls. These investigations also made possible the precise determination of the carotenoid pigments, because it was found that the rapid oxidation of the yellow pigments which takes place when the etiolated leaves are ground with sand and acetone during the extraction of the pigments may be prevented by treatment of the leaves with hot water (4). By the use of the improved methods of analysis, it was possible to determine accurately the effect of red light on the formation of the carotenoids in etiolated barley plants.

When etiolated barley seedlings are exposed to red light, both carotene and chlorophylls increase rapidly. The xanthophylls, comparatively large quantities of which are present in the etiolated barley seedlings, increase more slowly. Investigation of the pigments of etiolated barley seedlings, which had been impregnated with sucrose and kept in the dark, demonstrated that the presence of sucrose did not promote the formation of carotene or chlorophyll. Determination of the absorption spectra of lutein acetate and of beta-carotene demonstrated that lutein and beta-carotene, the principal constituents of the leaf carotenoids, absorb considerable light in the red region of the spectrum in which chlorophyll exhibits maximum absorption. Since etiolated barley seedlings contain extremely little green

pigment, the red light absorbed by the yellow pigments in these plants must represent a large proportion of that absorbed by all the pigments that are present. Whether the formation of the carotene in red light is dependent upon the absorption of light by the yellow pigments or upon other factors remains to be established.

Experimentation

Plant material

Etiolated barley seedlings were obtained by germinating barley seeds in 6-inch square boxes in the dark room. All the seedlings were grown in the same soil in order to insure comparable nutritional conditions.

Apparatus

The apparatus for the illumination of the seedlings was constructed from a metal can $13 \times 9 \times 9$ inches. Light of the desired wave length was admitted to the can through two $6\frac{1}{2}$-inch square glass filters set in modeling clay over openings in the top of the can. A 100-watt, inside-frosted tungsten lamp was placed 3 inches above each of the glass filters. Ventilation for the light chamber was provided by means of flexible metal tubing $1\frac{1}{2}$ inches in diameter and $3\frac{1}{2}$ feet long soldered to openings in each end of the can. One tube was bent upward in the shape of an S; the other was bent downward

TABLE I

Transmission of light by red glass filters

Filter I		Filter II	
Wave length	Transmission	Wave length	Transmission
mμ	%	mμ	%
590	0.00	627.5	0.00
595	0.17	630	0.14
600	1.8	632.5	0.27
605	9.7	635	0.87
610	24.4	637.5	2.28
615	39.7	640	6.1
620	48.9	642.5	12.5
625	55.4	645	21.7
630	58.8	647.5	32.4
640	61.6	650	43.8
650	63.8	655	58.3
660	65.8	660	64.8
670	65.3	670	71.5
680	67.2	680	72.4
		690	73.3
		700	74.4

in the shape of an *S*. In order to prevent direct admission of light to the tubing, black baffles, 6 inches square, were placed 3 inches from the open ends. Temperature within the light chamber varied from 21 to 26.6°.

Typical spectral transmissions of the two types of glass filters used to obtain red light are shown in table I. These values were subject to some variation due to imperfections in the surface and to variation in the thickness of the glass. The transmission curves and the absorption of light by solutions of the pigments extracted from the leaves were determined with the photoelectric spectrophotometer described by SMITH (**2**).

In order to make certain that the etiolated plants were exposed only to light of the wave lengths transmitted by the red filter, blue glass filters were placed over the red glass filters, and etiolated plants were grown in the light chamber while the tungsten lamps were allowed to burn continuously. After several days, the plants did not contain increased quantities of carotene or green pigments. This indicated that stray light was not entering the light chamber and that blue light was not transmitted by the red glass filters.

DETERMINATION OF PIGMENTS

Samples of the leaves (5 gm.) were placed in water at 95–100° for 1 minute. The water was separated by decantation and the leaves were ground with sand (12.5 gm.) and a little acetone. The ground mass was placed on a filter consisting of heat-treated siliceous earth supported on cotton, and the solvent was removed from the leaf material with suction. The residue was then washed with acetone until all the pigments had been removed. After the addition of ether (75 ml.) to the filtrate containing the pigments (75 to 100 ml.), the pigments were transferred to the ether by further dilution of the solution with water (about 200 ml.). The aqueous layer was separated from the ether and re-extracted with fresh ether (40 ml.). The combined ether layers were washed well with water and rinsed into a volumetric flask (100-ml.) with ethanol. An aliquot portion of this solution was diluted several times with ethanol, and the absorption of the solution was determined. Another aliquot portion of the solution of pigments was treated with a solution of potassium hydroxide in methanol in order to saponify the chlorophylls. The saponified chlorophylls were separated from the yellow pigments by washing the solution with water after the addition of some ether. The ether solution of the carotenoids was transferred to a volumetric flask and diluted to volume with ethanol. An aliquot portion of this solution was diluted with ethanol and the absorption was determined. Another aliquot portion of the solution of the carotenoids was diluted with petroleum ether, and the pigments were transferred to the petroleum ether by the addition of water to the solution. The petroleum ether solution was then extracted successively with 60, 70, 80, and 90 per

cent. methanol in order to remove the ether and the xanthophylls. The petroleum ether solution of the carotenes was diluted to a definite volume with ethanol, and the absorption was determined.

Experience with the leaf xanthophylls had demonstrated that leaves contain a mixture of these pigments which differ from one another with respect to their spectral absorption properties (3). In order to compare the xanthophyll, carotene, and chlorophyll content of leaves which had been permitted to develop under various conditions, it was necessary to calculate the spectral absorption coefficients of the pigments per gram of leaf material per liter of extract. In order to prevent confusion with E, the symbol for the absorption coefficient per gram of pigment per liter of solution, the following symbols have been used:

$$s = (\log I_o - \log I)\ (1/Lc)$$

where I_o and I are respectively the transmission of L cm. (2 cm.) of solvent and solution. The concentration, c, is expressed in grams of leaf material per liter of extract. Since s(total pigments), s(total carotenoids), and s(carotene) were determined experimentally,

$$s(\text{chlorophyll}) = s(\text{total pigments}) - s(\text{total carotenoids}),$$

and

$$s(\text{xanthophyll}) = s(\text{total carotenoids}) - s(\text{carotene}).$$

Results

The results of several experiments are summarized in table II. In experiment I, the pigments of etiolated barley seedlings 12 days old were determined. In experiment II, etiolated barley seedlings 5 days old were exposed continuously for 89 hours to light from filter I. For experiment III, the seedlings were exposed continuously for 168 hours to light from filter I. The plants for experiment IV were exposed continuously for 48 hours to light which passed through two glasses of filter II. In experiment V, the etiolated seedlings were exposed continuously for 96 hours to light which passed through two glasses of filter II.

The pigment content of etiolated barley seedlings which had been impregnated with 7.5 per cent. sucrose solution is recorded under experiment VI in table II. The etiolated seedlings, 9 days old, were cut so that the leaves were about 3 inches long. These leaves were impregnated with the sucrose solution by alternately exposing the leaves to vacuum in the presence of the sugar solution and then releasing the vacuum. The impregnated leaves were permitted to stand in the dark with their cut ends in a little of the sucrose solution. After 15 hours the leaves were removed from the solution, killed in hot water, and the relative proportions of the carotenes and xanthophylls were determined.

TABLE II

FORMATION OF PIGMENTS IN ETIOLATED BARLEY SEEDLINGS EXPOSED TO VARIOUS CONDITIONS

Wave lengths	Pigments	Absorption coefficients in experiments					
		I	II	III	IV	V	VI
mμ		*s*	*s*	*s*	*s*	*s*	*s*
440	Carotene	0.0010	0.0085	0.0078	0.0068	0.0071	0.0016
	Xanthophyll	0.0177	0.0260	0.0239	0.0199	0.0196	0.0217
	Chlorophyll	0.0003	0.0415	0.0360	0.0248	0.0311	.....
455	Carotene	0.0010	0.0096	0.0087	0.0078	0.0081	0.0016
	Xanthophyll	0.0139	0.0209	0.0188	0.0157	0.0158	0.0177
	Chlorophyll	0.0001	0.0195	0.0182	0.0101	0.0155	.....
465	Carotene	0.0009	0.0083	0.0076	0.0066	0.0068	0.0013
	Xanthophyll	0.0138	0.0214	0.0189	0.0166	0.0159	0.0174
	Chlorophyll	0.0000	0.0208	0.0192	0.0110	0.0161	.....
475	Carotene	.....	0.0087	0.0077	0.0069	0.0071	0.0014
	Xanthophyll	.....	0.0217	0.0196	0.0171	0.0163	0.0196
	Chlorophyll	.....	0.0154	0.0130	0.0070	0.0118	.....
662	Chlorophyll	.....	0.0467	0.0437	0.0301	0.0373	.....
664	Chlorophyll	0.0000	0.0473	0.0447	0.0302	0.0375	0.0000
666	Chlorophyll	.....	0.0453	0.0422	0.0295	0.0356	.....

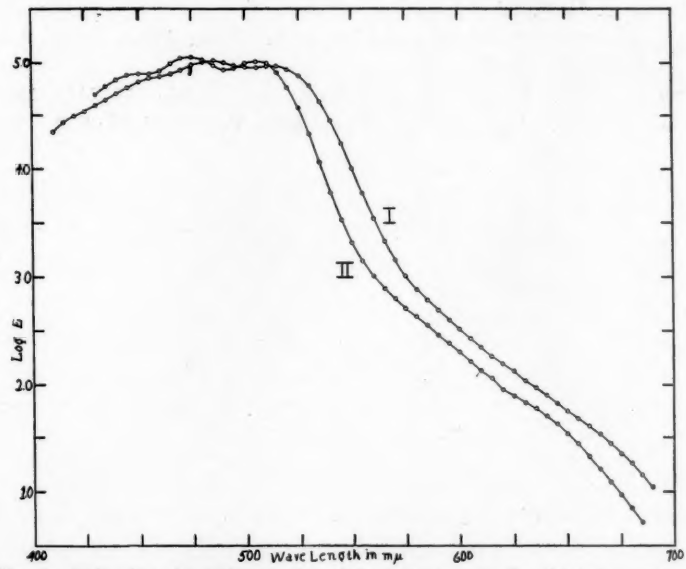

FIG. 1. Molecular absorption spectra of β-carotene, curve I; of lutein acetate, curve II. Solvent is carbon disulphide.

Molecular, spectral absorption curves of beta-carotene and of lutein acetate in solution in carbon disulphide are shown in figure 1. Since it was impossible to obtain a sufficiently concentrated solution of lutein in carbon disulphide for the determination of the absorption coefficients in the red region of the spectrum, it was necessary to use the more soluble acetate. This ester of lutein exhibits molecular absorption coefficients identical with those of lutein in the spectral region from 400 to 530 mμ. The molecular absorption coefficients were calculated according to the formula:

$$\log E = \log [(\log I_o - \log I)\ (1/Lc)]$$

where the concentration, c, is expressed in moles per liter of solution. Even considering the shift in the absorption spectra of the carotenoids caused by carbon disulphide (**2**), these pigments absorb considerable light in the red region of the spectrum in which chlorophyll exhibits maximum absorption.

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A PHOTOELECTRIC PHOTOMETER FOR CHEMICAL ANALYSIS

B. N. Singh and N. K. Anantha Rao

(WITH THREE FIGURES)

Introduction

Colorimetric methods have in recent years assumed considerable importance in chemical analytic procedures. The advantages inherent in these methods are their extraordinary simplicity, expeditious nature, and the possibility of measuring quantitatively with their aid concentrations which are measurable only with great difficulty, if at all, by gravimetric methods. On the other hand certain inherent imperfections of the method need elimination. Some of the sources of error are the disparities in color of standard solutions, the necessity for dilution at higher concentrations, and the dependence on the ability of the eye to match varying depths of color, an operation in which the human eye is notoriously inefficient. Moreover color blindness, a defect not uncommonly met, stands as a serious draw-back in the use of these methods.

The frequent necessity of making a large number of quantitative estimations of sugars and other substances in physiological researches has repeatedly called for simplifying the procedure so as to eliminate the possibilities of personal error in observations, to detect more minute differences than is possible by preceding methods, and to be applicable to varied purposes. With this object in view the apparatus described here was developed in this laboratory and has been in use for some time.

Principle

The general principle of the apparatus is similar to the one that has been developed in this laboratory for chlorophyll estimation (**3**). In devising this apparatus for the chemical analysis of sugars and other organic and inorganic substances, use has been made of the principle of determining the concentration of the substance occasioning a definite color reaction by the measurement of light absorption in the solution within an appropriately selected region of the spectrum. Following the well-known Lambert-Beer law—"The negative logarithms of the transmitted light intensities K (extinction coefficient) at equal stratum thickness bear the same proportion to each other as the concentrations, C, of the dissolved substances,"—the concentration in any particular case is calculated from the equation $C_2 = C_1 \frac{K_2}{K_1}$. When once a series of measurements is recorded *in the same region of the spectrum,* on a suitably graduated range of solutions having known concentrations, a calibration graph is obtained from which the con-

centration corresponding to any observational reading may then be read off directly.

Apparatus

The apparatus is shown in figure 1. A constant source of light, L, supplies two equal beams of light which fall on the absorption vessels, V_1 and V_2. The transmitted lights from the absorption vessels pass through the

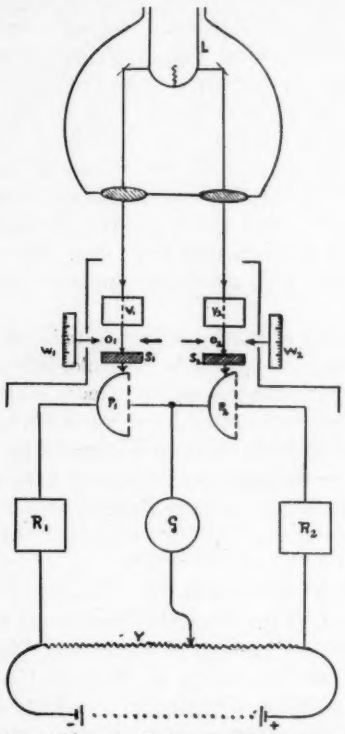

FIG. 1. Diagram of photoelectric photometer.

openings, O_1 and O_2, whose sizes can be measurably varied. S_1 and S_2 are two slots for the reception of the spectral filters within the range of which a particular measurement is to be made. The transmitted lights from the two sides then fall on the two photoelectric cells, P_1 and P_2, respectively (Osram photocell, Caesium. C. G. I. Becker and Co.), which emit currents in direct proportion to the light energies absorbed on their faces. The photoelectric emission produced by two beams of the same quality and quantity is the same, and hence if the quality is unchanged and the quantity alone varies, the rate

of current emission would be proportional to the quantity of light. A compensating device which converts direct reading into null-method is employed, the balance being effected by adjusting the lights that alter the photoelectric currents. The circuit is essentially of the bridge type, R_1 and R_2 being the protective resistances (10,000 ohms), and G a sensitive null-point galvanometer. The differences, if any, in the two cells are eliminated by the use of the common battery shunted by r.

The *caesium cell* is specially selected on account of its even sensitivity in response to light over the whole visible range.

Figure 2 illustrates the light diminution arrangement. Each of the

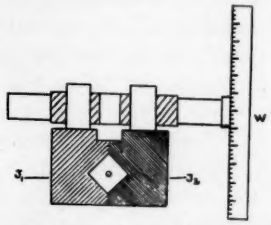

FIG. 2. Arrangement for diminution of light.

openings, O_1 and O_2, are formed by two rectangular V-shaped jaws, J_1 and J_2. The latter can be moved, one above the other, either towards or away from each other, in such a way that the centre of the aperture always coincides with the optic axis. The ratio of the two amounts of light transmitted is proportional to the ratio of the two areas of the apertures. The graduations[1] etched on the recorder, W, give a measure of the transmitted light as a percentage of the incident light. The scale on the recorder represents 100 as the largest opening and 0 the smallest.

The measurement is made within the range of a narrowly defined region of the spectrum which is furnished by selective spectral filters, dividing the visible spectrum into eight equal parts in terms of wave-lengths (750mμ, 690mμ, 610mμ, 570mμ, 530mμ, 500mμ, 460mμ and 430mμ). To make the values obtained with the different spectral filters comparable, corrections are made by comparing each filter in turn with one (430mμ) taken as the standard. The transmission value should be multiplied by the correction factor (obtained as above) for the filter used.

[1] If D = diagonal of the square aperture (O_1, O_2), d = the variable diagonal between 0 and D, a = value of the angle between 0 and 360° through which W turns, and i = intensity of the light (the area of the aperture) then

(1) $i = \left(\frac{d}{\sqrt{2}}\right)^2 = \left(\frac{a}{360°}\right)^2$ for d from 0 to D, for i from 0 to 1, and for a from 0 to 360°, and

(2) $a = \sqrt{i \cdot 360°} = 0$ up to 360°, for i from 0 to 1.

Procedure and accuracy

It is first necessary to determine the extinction of a solution of known concentration in order to be able to calculate in a simple manner any desired concentration from the amount of its extinction *in the same region of the spectrum.*

The filters required for the measurement are introduced one on each side (S_1 and S_2). The two beams of light are then adjusted to equal intensity as indicated by the null-point. For obtaining this, r may be used as a fine adjustment. The colored solution of the substance in a known concentration is filled into an absorption vessel (V_1) of suitable stratum thickness[2] and brought into the path of one of the beams of light. An absorption vessel of similar stratum thickness is filled with the solvent[3] (treated in exactly the same way as the solution) and brought into the path of the other beam of light. This compensates absorption in the solvent and the loss of light on the sides of the absorption vessels. More light is now absorbed by the solution (V_1) than the solvent (V_2) and thus the photoelectric currents become unequal. Consequently the bridge is thrown out of balance and this is indicated by the deflection of the galvanometer. The recorder, W, on the side of the solvent is now slowly turned in, *i.e.*, the transmitted light on this side is diminished until the null-point is again restored. After noting the value, the absorption vessels and the contents are interchanged and a second measurement taken. This compensates variations in the wall thickness, if any, of the absorption vessels. From the average of the readings, the extinction coefficient is extracted. This preliminary experiment gives K_1 and C_1. The test solution is then estimated as above and the value for K_2 is obtained. Knowing C_1, K_1, and K_2, C_2 is easily calculated from the equation.

A brief account of the practical procedure of sugar estimation with the above apparatus following BENEDICT'S picric acid method (**1**) is described in the following paragraphs.

The sugars are extracted from the plant material by the method of DASTUR as followed by PARIJA and SARAN (**2**). The material is boiled in water for over 30 minutes, and is then thoroughly crushed in a mortar till a very fine paste is obtained. The water in which the leaves are boiled is used in making up the volumes of the pastes. The filtrate which contains the sugars is freed from impurities by adding lead acetate and subsequent deleading. The final filtrate is concentrated on a water bath and again filtered for the last time. Two cc. of this sugar solution are taken in a test-tube marked at 12.5 cc. and 25 cc. To the 25-cc. mark the picrate picric acid reagent is added, left for a few minutes and the whole thing filtered. Eight cc. of the filtrate are poured into a similar test-tube and 1 cc. of 20 per cent. sodium

[2] To be determined according to the experiment.
[3] With clear aqueous solutions this vessel is filled with distilled water.

carbonate solution is added. The test-tube is then plugged and immersed in boiling water for 10 minutes, cooled, and diluted to the mark. A brown color is obtained owing to the presence of the reducing sugars.

An equal quantity of distilled water is then treated in the same manner as the sugar solution.

The measurement is then made as described above. From the reading obtained, the concentration is read off directly from a calibration curve (fig. 3).

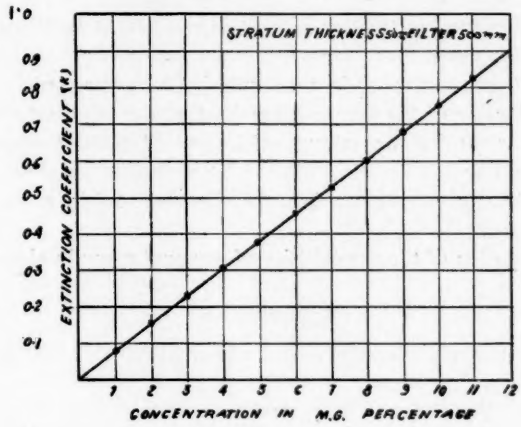

FIG. 3

The determination of K, and thus also the determination of the concentration, becomes more accurate the greater the absorption of the solution under investigation. Hence accuracy can be increased by increasing the stratum thickness, as can be seen from table I.

TABLE I

ESTIMATION OF A SINGLE SAMPLE OF GLUCOSE (0.4 GRAM PER LITER) USING DIFFERENT STRATUM THICKNESSES

STRATUM THICKNESS	TRANSMISSION	EXTINCTION COEFFICIENT (K)	PERCENTAGE ERROR IN CONC. (C)
mm.	%		%
5	90	0.046	± 4.96
10	70	0.155	± 3.35
30	50	0.301	± 2.14
40	30	0.523	± 1.43
50	20	0.699	± 1.22
60	10	1.000	± 1.035
70	5	1.301	± 0.817

For general purposes a stratum thickness which gives a transmission value between 50 per cent. and 10 per cent. can be chosen. Similarly, a filter which gives the smallest transmission value for the solution increases the accuracy of the estimations. In cases of divergencies from the Lambert-Beer law, evaluation with the aid of a calibration curve is to be resorted to. With the above conditions satisfied, an accuracy of ± 2.24 per cent. is easily obtainable in practice.

Some of the special advantages of the apparatus are:

(1) Elimination of the use of standard solutions.

(2) Compensation of the loss of light in the solvent and on the windows of the absorption vessels.

(3) Results are arrived at by exact physical measurements.

(4) Elimination of the necessity for diluting the solutions at higher concentrations by changing the stratum thickness.[4] Intensely colored solutions may be measured without being diluted by reducing the strata down to a few μ, while faintly colored solutions may be measured by employing strata of greater depths.

(5) Elimination of the possibilities of personal error in observation.

Applications

The apparatus can be employed for varied purposes. The chief of them are:

(1) For determination of concentrations.

(2) For obtaining absorption curves: the measurement is made using all the filters, and the extinction coefficient (K) values thus obtained are plotted against the wave lengths. Such curves characterize the absorption properties of the substance for the particular concentration used.[5]

(3) For obtaining color curves: the logarithms of the extinction coefficients (K) are plotted against the wave lengths. The form of these curves is independent of the concentration and stratum thickness and unambiguously characterizes the nature of the absorption of the substance in solution.[5]

(4) For checking the purity of solutions: if the test solution is quite pure the logarithms of the extinction coefficient (K) obtained with various filters maintain a certain constant relation to one another, *i.e.*, the color curves run parallel to each other. This is of importance in extraction and purification of substances.

The apparatus has also been successfully employed in this laboratory for

[4] The transmission is dependent upon stratum thickness according to the Lambert-Beer law.

[5] (2) and (3) are employed in the study of the spectro-photometric properties of substances. A detailed account of the spectro-photometric properties of chloroplast pigments will appear elsewhere shortly.

the determination of nitrogen with Nessler's reagent, and of phosphorus as phosphomolybdate. In all these cases, as well as for other methods of organic and inorganic estimations the procedures detailed by YOE (**4**) for colorimetry are applicable with the necessary modifications.

Summary

1. A photoelectric apparatus for chemical analytic purposes is described.

2. The principle of the apparatus is the measurement of light absorption of a solution within a narrowly defined region of the spectrum with the aid of a measurably variable light diminution.

3. The special advantages and applications of the apparatus are given.

4. The accuracy is $\pm$ 2.24 per cent.

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BRIEF PAPERS

APPLICABILITY OF KUNDT'S RULE TO CHLOROPHYLL

G. MACKINNEY

(WITH ONE FIGURE)

KUNDT's rule is frequently quoted, either in reference to the state of chlorophyll in the living leaf (3), or in comparing absorption spectra of chlorophyll preparations in different solvents (6). Its use in these respects is usually based on a determination of the wave length of maximal absorption for the main red band. Certain observations (5), indicating an apparent shift in the position of this maximum, may be explained on the basis of different degrees of skewness of the chlorophyll absorption band, and because of the relevance of these observations to KUNDT's rule, evidence is reviewed, denying the rule either general significance or specific applicability to chlorophyll.

As summarized in 1878 (4), KUNDT's rule is a statement (Satz): "Man kann mithin als Resultat der ganzen Untersuchung den folgenden Satz aussprechen: Hat ein farbloses Lösungsmittel ein beträchtlich grösseres Brechungs- und Dispersions-vermögen als ein anderes, so liegen die Absorptionsstreifen einer in den Medien gelösten Substanz bei Anwendung des ersten Mittels dem rothem Ende des Spectrums näher als bei Benutzung des Zweiten." HOUSTOUN (2) writes of this (p. 331): "KUNDT in 1874 dissolved substances that produced absorption bands, using different solvents, and found that the position of the band varied with the solvent." HOUSTOUN then quotes KUNDT's conclusions and he comments: "This statement is not true; the band moves as often the one way as the other, and generally there is a marked change in intensity and shape, in comparison with which the shift in position can be neglected." Discussing anomalous or "selective" dispersion, HOUSTOUN has derived formulae relating the indices of refraction, N (of the solution), n (of the solvent), and the absorption coefficient k, as a function of wave length, λ. If λ_o represent the wave length of free period of vibration of an electron, then absorption will be encountered for the condition $\lambda \rightarrow \lambda_o$. It does not seem possible that one may predict, *a priori*, for a hitherto unused solvent of refractive index n_1, whether N, λ_o, or both will be affected, nor whether they will necessarily follow the direction indicated by KUNDT's rule.

KUNDT's rule is ordinarily applied to chlorophyll by considering the wave length of maximal absorption as a function of the refractive index of the dissolving medium. In most organic solvents, this maximum lies between 6550 and 6700 Å, while for colloidal chlorophyll, it is close to 6800 Å, and

for chlorophyll *in vivo*, between 6800 and 6820 Å. The curve relating these quantities is then extrapolated to 6800 Å, and on the assumption that KUNDT's rule is obeyed, it is concluded that a medium of abnormally high refractive index would be required, and that therefore chlorophyll cannot be in true solution in the chloroplast. The concomitant requirement of KUNDT, "ein beträchtlich grösseres Dispersions-vermögen," has been largely overlooked, with one notable exception. Thus SORBY (8) also noted that the exact position of the absorption bands of chlorophyll varied considerably according to the solvent, even though inert. "I have carefully determined the order in which various liquids thus raise bands, and compared it with the order of their specific gravity, and of their refractive and dispersive power, but have hitherto failed to recognize any simple connexion between what may be called their *band-raising power* and any other physical property." (Italics as in the original.)

The extensive measurements of BIERMACHER (1) on the pure components of chlorophyll indicate several exceptions to the rule. The writer has also found a specific example of failure, for the blue component, in acetone N_D 1.35886, and in dichloroethane N_D 1.45026. The maxima, determined spectrophotoelectrically, lie at 6630 Å $\pm$ 5 Å, and the shapes of the two curves are virtually identical over the whole band for these two solvents.

In denying, therefore, a specific applicability of the rule to chlorophyll, we may briefly examine the causes of certain apparent shifts in the position of maximal absorption. When comparisons are made, either in different solvents, or for different preparations, the degree of skewness may introduce an appreciable error in the location of the maximum. The band "axis" determined by visual or photographic methods, may not coincide with the maximum. A study of the absorption for the whole band is therefore desirable, prior to locating the position of the maximum. Visual photometry yields results that are reproducible within quite narrow limits, certainly within $\pm$ 15 Å, for a given observer. SMITH (7) has taken into account the visibility curve in explaining certain discrepancies, between different laboratories. The reasoning may be extended for chlorophyll to show that in the red portion of the spectrum, where within 200 Å, the absorption coefficient for chlorophyll may change by two orders of magnitude, or more, and the visibility function is rapidly approaching zero, the results, however reproducible, may be peculiarly unreliable, particularly in view of the skewness of the band, caused by the introduction of the *b* component, and the superposition of its absorption on that of chlorophyll *a*. This has been indicated (5) in a comparison made between pigments from leaves and from Sultanina grapes, where the visually determined maxima did not agree within 50 Å, because of an illusory displacement of the true maximum. This is further illustrated in figure 1, where two curves are shown, determined

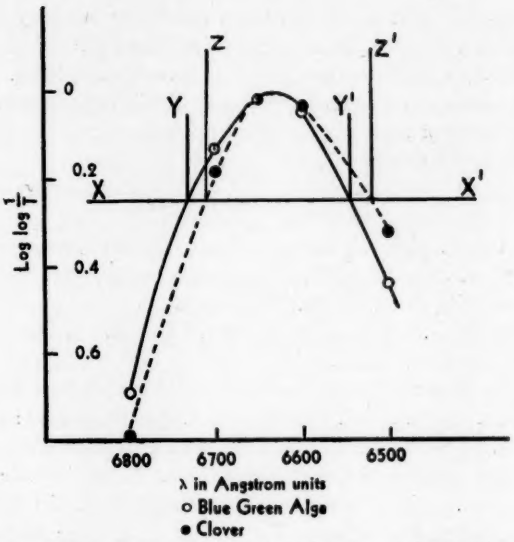

FIG. 1. Curves illustrating the observed band limits of chlorophyll preparations (for explanation see text).

spectrophotoelectrically, both in 80 per cent. aqueous acetone, one for the pigments from a blue green alga, deficient in chlorophyll *b*, the other for clover, considerably richer in this component. If we use a visual instrument and arbitrarily set the photometer at a given transmission level, we may plot an appropriate function of the transmission (in this case, the double logarithm of the reciprocal), as a line, designated XX^1 in the figure, and the observed bands will have as limits the intercepts of the curves with this line, namely YY^1 and ZZ^1, repectively. As the line XX^1 is moved up the Y ordinate, the observed difference is minimized but not in practice entirely eliminated.

Conclusions

The degree of skewness of the absorption curve for chlorophyll solutions (which may be caused by a change either in the dissolving medium or in the ratio of the two components) may introduce an appreciable error in the location of the wave length of maximal absorption. Because of the relevance of these observations to KUNDT'S rule, his original statement has been examined. It is of interest that SORBY, contemporary with KUNDT, came to directly opposite conclusions. In justice to KUNDT, no law was formulated.[1]

[1] Although not strictly germane to the subject of this note, KUNDT'S attitude is reflected in the following passage (4b): "Der Nachweis, in wie weit die sämmtlichen oben

His statement has been found on more searching scrutiny to contain too many exceptions, and receives no theoretical support. With specific reference to chlorophyll, BIERMACHER has noted several exceptions, and the writer has found a similar case with acetone and dichloroethane as solvents.

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dargelegten Anschauungen wirklich in Uebereinstimmung mit der Natur sind, und ihre weitere Ausarbeitung wird die Aufgabe von Specialuntersuchungen sein müssen. Bis diese vorliegen, wird es gut sein noch andere Speculationen, die man allerdings mit Leichtigkeit an das Vorstehende anknüpfen könnte, zu unterlassen.''

NOTES

Summer Meeting, Ottawa, Canada.—The summer meeting at Ottawa, Canada, to be held June 28–30, 1938, in connection with the A.A.A.S. summer meeting, offers many attractive features. Several symposia are being organized, in addition to the general program, and the inspection trips and demonstrations at the Experimental Farm and National Research Laboratories. The suggested symposia topics include *Microelements and Deficiency Diseases*, to be held jointly with phytopathologists; and *Drought Relations*, held jointly with geneticists.

The arrangements are being guided by Dr. G. W. SCARTH, of McGill University, chairman of the program committee for this meeting. Collaborating with him as members of the committee are Mr. B. M. DAVIS, Division of Horticulture, Experiment Farm, Ottawa; Dr. ROBERT NEWTON, National Research Council, Ottawa; Dr. R. D. GIBBS, Department of Botany, McGill University; and Dr. F. P. CULLINAN, *ex officio*. The headquarters have been arranged at the Chateau Laurier, Ottawa. This event provides opportunity for a lively exchange of ideas with our Canadian colleagues, and for a visit to the Canadian seat of government under the most auspicious circumstances. Those who were fortunate enough to attend the meetings at Toronto in 1921, and 1924 (British Association), know the lavish hospitality of Canadian hosts. There should be a large attendance from all sections of North America.

Richmond Meeting.—The program committee for the Richmond meeting in December, 1938, is constituted as follows: Dr. W. R. ROBBINS, New Jersey Agricultural Experiment Station, chairman; Dr. H. H. ZIMMERLEY, Virginia Truck Experiment Station, Norfolk, Virginia; and Dr. DANIEL G. CLARK, Cornell University. The secretary-treasurer, Dr. F. P. CULLINAN, is *ex officio* member of the committee. This meeting will be held December 27–29, 1938; those who plan to appear on the programs should make plans early, so that the program committee can organize the papers into suitable groups, and provide commodious rooms for our meetings. Close cooperation with the committee in all matters pertaining to the arrangements is necessary for most effective service.

Western Section.—The Western Section of the American Society of Plant Physiologists will meet with the Pacific Division of the A. A. A. S. at San Diego, Tuesday to Friday, June 21–24, 1938. Headquarters will be in Balboa Park, the site of the 1915–1916 and 1935–1936 San Diego Expositions. No hotel has been designated as official. The leading hotels of the city are within 15 minutes walking distance of the grounds.

The tentative program consists of sessions for submitted papers on Tuesday, Thursday, and Friday mornings; a joint symposium with the Western Society of Soil Science on *Salt Tolerance of Plants and Related Problems*, Dr. F. M. EATON, chairman, on Tuesday afternoon; a joint symposium with the section on botanical science on *Plant Invasion on the Pacific Coast* on Wednesday morning; a joint symposium with the Pacific Section of the Botanical Society on *Cell Wall Structure*, Dr. O. L. SPONSLER, chairman, on Wednesday afternoon; and a joint symposium with the Pacific Section of the Botanical Society on a *Résumé of Progress in Plant Science* on Thursday afternoon. A biologists' dinner is scheduled for Wednesday evening, and the present plans call for a plant physiologists' dinner on Thursday evening.

George James Peirce.—The American Society of Plant Physiologists, as a mark of its esteem and affection for him, has dedicated this number of PLANT PHYSIOLOGY, in celebration of his seventieth birthday anniversary on March 13, 1938, to Dr. GEORGE JAMES PEIRCE of Stanford University. Dr. PEIRCE was born at Manila, Philippine Islands, son of GEORGE HENRY and LYDIA ELLEN (EATON) PEIRCE. Following his preliminary education, he entered the Lawrence Scientific School of Harvard University from which he received his degree of Bachelor of Science in 1890. Two years later he was awarded a fellowship at Harvard which permitted him to go abroad, and he spent two years, 1892–1894, at Bonn, Munich, and Leipzig. His higher degrees, M.S. and Ph.D., were won at Leipzig in 1894.

During the first two years following his European study, he was assistant professor of botany at Indiana University. In 1897 he married ANNA HOBART of Cincinnati, and went to Stanford University where he found his life work. He was assistant professor of botany there from 1897 to 1900, associate professor to 1910, professor since 1910, and now emeritus professor at Stanford. He has collaborated in various public services, with the United States Forest Service, the Department of Justice, the Palo Alto Board of Health, the Red Cross, and the California Fuel Administration during the World War. He is a member of many societies, both American and foreign, has written several text-books on plant physiology and biology, and has been honored with the presidency of the California Botanical Society (1931), and the Botanical Society of America (1932). The American Society of Plant Physiologists honored him at the Des Moines meeting in 1929 by selecting him as a CHARLES REID BARNES life member of the society. It is a pleasure at this time to extend to him the affectionate and cordial greetings and good wishes of the Society for many more years of activity, with health and strength to enjoy them.

Errata.—Additional errors in the first three numbers of volume 12 of PLANT PHYSIOLOGY have been reported to the editor, and they are listed in

the hope that members will make corrections in their copies of the journal. Thanks are due to Dr. W. F. LOEHWING and his students for aid in this connection. In most instances the authors have been consulted, also.

P. 207, line 3, for (**8**) read (**2**).
P. 253, line 7 from bottom, for "(**11**)" read (**9**).
P. 337, line 5 from bottom, for "obesrve" read observe.
P. 506, line 2, for "(**10**)" read (**9**).
P. 529, table I, column 7, item 3, for "1.252" read 0.798.
P. 441, line 9, for "(**1**)" read (**11**).
P. 674, line 14, for "definie" read define.
P. 438, reference no. 61, for "ONUNUKI" read OKUNUKI. (This reference deals with auxin b, not auxin a or heteroauxin.)

Cell Necrobiosis and Protoplasmic Death.—Dr. W. W. LEPESCHKIN is the author of a monograph entitled *Zell-Nekrobiose und Protoplasma-Tod,* Protoplasma Monographien, zwölfter Band, pp. 198, inclusive of bibliography, published by Gebrüder Borntraeger, Berlin. In this monograph of approximately 145 pages of text LEPESCHKIN brings together for the scientific reader all of the essential evidence which has been presented in the literature on the subject of cell necrobiosis. The clear distinction which he makes between the death of a many-celled organism and the death of its constituent cell units is one which has not been brought emphatically to the attention of biologists before. While it is well known to scientists that the organism may die and its constituent cells go on living for some time afterward, it is scarcely appreciated by what progressive steps death in the individual cell really takes place. Dr. LEPESCHKIN'S monograph therefore concerns itself with the progressive steps in the dying of the individual cell, which constitutes cell necrobiosis.

In order to make this clear he discusses in the first part of the monograph and brings to bear thereon the evidence in the literature of the manner in which different agents bring about the death of the cell, as well as the discernible changes which take place in the cytoplasm and in other parts of the cell during the processes of gradual dying. For example, he discusses the morphological changes during cell necrobiosis which take place in living matter, as well as the cytological changes, purely physical changes, chemical changes, and the setting free of energy. In connection with these subjects, LEPESCHKIN discusses his own Vitaid Theory which is well known to those who are conversant with the literature of general physiology.

In the second part of the monograph he discusses special cases of cell necrobiosis and the death of protoplasm. In this he takes up for consideration mechanical influences on living matter, and likewise the influences of extreme heat, extreme cold, desiccation, the influence of radiant energy, and

of acids and bases, as well as of salts and narcotics. The monograph is accompanied by a thoroughgoing bibliography of approximately forty pages and by an author index.

In producing this work Dr. LEPESCHKIN has placed the scientific world much in his debt. With the exemplary clarity and brevity which characterize all of his papers, he gives a picture of his subject which cannot help but be of the greatest interest and importance to every biologist. It is a pleasure to congratulate Dr. LEPESCHKIN on this achievement.—C. B. L.

Colloid Chemistry.—During the early part of the year 1935–1936, the George Fisher Baker lectureship in chemistry at Cornell University was held by Dr. R. A. GORTNER of the Agricultural Biochemistry department of the University of Minnesota. The lectures were published in 1937 by the Cornell University Press under the title *Selected topics in colloid chemistry with especial reference to biochemical problems.* The eight topics selected for discussion are as follows: the beginnings of the science; what is colloid chemistry; some basic concepts; some fundamental properties of colloid systems; electrokinetics; surface tension, surface energy, interfacial tension, and molecular orientation; adsorption; and the water relationships of bio-colloids. Having experienced the pleasure of listening to Dr. GORTNER'S lectures on colloidal chemistry at Minnesota, the reviewer knows how intensely interesting were these lectures at the time of their delivery. They lose little of their fire and enthusiasm by appearing in printed form. Students will find them delightfully informative and stimulating. They are of greatest value to students of biology who may not always appreciate the pervading importance of the colloidal state in all physiological processes. The book is recommended as part of the required reading in courses in plant physiology. It is priced at $2.50 per copy, and may be obtained from the Cornell University Press, 124 Roberts Place, Ithaca, New York.

Determination of Amino Acids.—The methods of quantitative determination of the individual amino acids have been brought together in a valuable book by Dr. RICHARD J. BLOCK, of the New York State Psychiatric Institute and Hospital. It is published in mimeoprint form with flexible binding by the Burgess Publishing Co., Minneapolis, Minnesota. The methods are presented in eleven chapters, the first five of which deal with groups of acids, as follows: arginine, histidine, and lysine; tyrosine, tryptophane, dihydroxyphenylalanine, and thyroxine; proline, and hydroxyproline; cystine, cysteine, and methionine; and glutamic, aspartic, and hydroxyglutamic acids. The next five chapters deal with single amino acids, alanine, glycine, leucine, phenyl alanine, and serine. The last chapter describes the general methods of separation of the amino acids in mixtures. It includes the meth-

ods of FISCHER, CHERBULIEZ, DAKIN, BRAZIER, and PRZYLECKI and KASPRZYK. These general methods are presented in outline form, diagrams, which should be easily followed. Each chapter closes with a bibliography of the original methods papers, from which the precautions and limitations of the methods must be gleaned by the investigator. The compilation is very helpful, and though plant physiologists have not fractioned their nitrogenous materials to show the individual amino acids involved in metabolism, and do not know the significance of each acid sufficiently to use such information in interpreting metabolic changes, yet we are approaching the necessity of making such detailed studies. To those engaged in studying the hydrolytic products of proteins and protoplasm, this book would be a great time saver. It also contains diagrams of some of the most useful pieces of apparatus needed for the separations. With indexes it contains 85 pages, and is priced at $3.00 per copy.

Wheat and Flour Quality.—A practical work on wheat and flour quality by Dr. C. O. SWANSON of the Kansas State Agricultural College has been published by the Burgess Publishing Co., Minneapolis, Minnesota. It contains 26 chapters, 227 pages, and will be very valuable to the entire milling industry. It is written in simple style, and some of the chapters are of interest to the physiologist, especially chapters VIII to XIV. These bear titles as follows: Whence the substances in wheat; influence of climate and soil on quality; soil nitrogen, the key element in protein content; factors that influence the quantity of protein; enzymes, the tools of life processes; the life activities of the wheat kernel; and germination and diastatic activity. The price of this interesting work is $4.00 per copy. It comes in mimeoprint with flexible binding, and may be ordered directly from the publishers.

Fertilizers.—The second edition of Dr. Firman E. Bear's *Theory and Practice in the Use of Fertilizers* has been published by John Wiley & Sons, Inc., New York. The rapid changes in the manufacture and use of concentrated fertilizers have made a rewriting necessary. In addition, the better knowledge of the function of trace elements in successful plant growth has made the inclusion of a chapter on trace elements in soils and crops necessary. The book is a useful introduction to the theories upon which soil amendment is based, and a practical guide to the user of fertilizers in the control of soil fertility and the improvement of crop yields. The book is quoted at $4.00 per copy.

Chemistry of Plant Constituents.—A summary of the problems of plant chemistry has been prepared by Dr. OLE GISVOLD and Dr. CHARLES H. ROGERS, both of the School of Pharmacy of the University of Minnesota, with the title: *The Chemistry of Plant Constituents.* It is published in mimeoprint form with flexible covers by the Burgess Publishing Co., Minneapolis, Minnesota, at $3.50 per copy. There are 15 chapters, the introductory chapter dealing with the functions of plant parts, and the inorganic constituents essential to metabolism. The succeeding chapters take up the nature and origin of the following groups of constituents: carbohydrates; vegetable fats; plant and insect waxes; phytosterols; proteins; alkaloids; glycosides; the anthocyanins and anthoxanthins; carotenoids; tannins; terpenes and oxygenated terpenes; vitamins; enzymes; and products of fermentation. Although there is much theoretical speculation involved in the attempt to depict the origin of plant constituents, such speculation is justifiable and necessary as long as definite information as to origins is lacking. There is a great deal of stimulating and valuable information crowded into the 295 pages of text, information for which plant physiologists will be thankful. An index makes browsing for specific information easy. Short bibliographies are used in connection with certain topics, but there is no general bibliography. It should find a hearty welcome among those interested in any phase of plant biochemistry.